REMARKS

This application has been amended in a manner that is believed to place it in condition for allowance at the time of the next Official Action.

Claims 29-30 and 33-36 are pending. Claims 29 and 34 have been amended. Claims 27-28 and 31-32 have been canceled. Claims 29 and 34 have been amended to recite that the male sterility is conferred by an artificial male sterility (AMS) gene. The term "both" has been added in claims 29 and 34 to recite that the plasmid vector contains at the same time the AMS gene and the transgene of interest, that are genetically linked. Claim 29 has been further amended by specifying that the therapeutic or prophylactic compound of human or animal origin is extracted and then administered in a human or an animal. Applicants further note that claim 29 recites that the method comprises transforming the nuclear genome of a plant. New claims 35-36 have been added. Support for the amended claims and new claims may be found in the original claims and in the present specification at page 6, line 19 to page 7, line 2; page 1, lines 8-13; page 14, lines 22-27; page 2, line 34; page 4, lines 17-24; and page 15, lines 6-8.

In the outstanding Official Action, claims 27-34 were rejected under 35 USC 112, first paragraph, as allegedly failing to comply with the written description requirement. In addition, claims 27-34 were rejected under 35 USC 112, first paragraph, for

allegedly not satisfying the enablement requirement. This rejection is respectfully traversed.

In imposing the rejection, the Official Action alleged that the specification was not enabling for an invention other than a method wherein the AMS gene is a glucanase or barnase gene, and the transgene encoding a therapeutic or prophylactic compound of human or animal origin is a dog gastric lipase gene.

However, the present specification teaches that the AMS gene may encode a protein which is able to degrade the RNA molecules (page 4, lines 16-22). In other words, any RNAse may be appropriate to confer artificial male sterility.

This is evidenced by the Vedel et al. article, cited in the prosecution of this application. Vedel et al. teach that AMS can be conferred by transforming plants with constructs containing a bacterial or fungal RNase gene (page 605, left column, lines 41-44). Specific examples of RNase genes are Aspergillus oryzae T1 TNase gene and barnase (page 605, right column, lines 3-71.) Vedel et al. also describe other AMS genes that include antisense expression inhibiting flavonoid expression, such as antisense of chalcone synthase gene (chs) (page 606, second paragraph), or the Rol c gene (page 606, third paragraph).

As further evidence of this assertion, applicants note that other examples of AMS genes are described in the European patent application EP 0 344 029.

The present application also teaches that a variety of genes can confer male sterility. These include RNase T1, barnase, endonucleases, proteases, isopentenyl transerase, glucanases, phospholipase A2, lipid peroxidases, gene encoding plant cell wall inhibitors or toxins, as well as antisense DNA or ribozymes.

Thus, the claimed method can be practiced by one skilled in the art with AMS genes other than barnase or glucanase. Accordingly we believe that the specification gives sufficient guidance to carry out the claimed method with any AMS gene.

The Examiner is also respectfully reminded that the claimed method is directed to preventing the transgene integrated in a nuclear genome of the plant to be disseminated in pollen. The gene encoding dog gastric lipase or collagen are examples of such a transgene. However, examples of mammalian proteins that have been expressed in plant are described in Lenee et al., which also has been cited by the pending Office Action (see col. 3, lines 3 to 21).

Upon reviewing the application, applicants submit that there is no objective technical reason or evidence to conclude that prevention of transgene dissemination would not be successfully achieved when a transgene sequence different dog gastric lipase or collagen is used. Accordingly, applicants submit that the specification provides sufficient guidance to claim transgenes other than dog gastric lipase or collagen.

The Office Action also alleged that the specification would not teach AMS, wherein mitochondrial transformation is performed. However, claim 29 recites that the nuclear genome of the plant is transformed with the AMS gene.

Therefore, in view of the above, we believe that the claimed invention satisfies the requirements of 35 USC §112, first paragraph.

Claims 27-34 were rejected under 35 USC 112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. This rejection is respectfully traversed.

The Official Action alleged that the terms "artificial male sterility gene" and "therapeutic or prophylactic compound" were indefinite.

However, as to the term "artificial male sterility gene", the term "artificial" does not characterize the gene per se but male sterility (see present specification, page 2, line 30). In other words, AMS denotes a male sterility which is induced in a plant by introduction and expression of a gene which confers male sterility, as recited in the specification.

Male sterility is thus called "artificial male sterility" because it results from a human transformation of the plant, by opposition with a male sterility that would be acquired without any genetic manipulation. By extension, the gene which is

used to confer artificial male sterility to the plant is called an "artificial male sterility gene" or AMS gene. In further support, the Examiner's attention is respectfully directed to page 2, lines 6-16 of the International Patent Application WO 96/26283. The publication also utilizes this term.

Applicants also believe that the term "therapeutic or prophylactic compound", applicants believe that the term is definite to one skilled in the art. As described in the present specification at page 4, lines 22-27, the present invention is concerned with a method for preventing the dissemination of a transgene from a plant which is cultivated for the production of compounds of therapeutic or prophylactic interest, i.e., compounds that are intended for use in humans or animals. The question of whether the therapeutic or prophylactic compound may be toxic or ineffective under some circumstances does not preclude the observation that the compound may nevertheless induce a therapeutic or prophylactic effect in the human or animals under other circumstances. As a result, applicants believe that while the terms may be broad, the terms are definite to one skilled in the art.

Thus, in view of the above, applicants believe that the claimed invention is definite to one skilled in the art.

Claims 29-30 and 33-34 were rejected under 35 USC 103(a) as allegedly being unpatentable over D'Halluin et al. in view of Metz et al. and Welter. This rejection is respectfully traversed.

As noted in the amendment of December 1, 2003, applicants believe that Welter fails to qualify as prior art. Welter was published after the priority date of the present application. Indeed, applicants are in the process of obtaining a certified translation of the French patent application FR 9702369 to comply with 37 CFR 1.55.

Nevertheless, applicants believe that Welter is not relevant in regards to the claimed invention. Welter describes the production of heterologous proteins in plants and is completely silent as to the use of artificial male sterility genes in the prevention of transgene dissemination.

D'Halluin et al. disclose a process for transforming monocotyledonous plant that comprises transforming plant cells with a sequence encoding a protein of interest and 3' transcription regulation signals (col 8, lines 37-45). The protein of interest may be a protein that renders the plant male sterile (col. 10, lines 32-50, in particular lines 39-42).

Selectable markers such as a kanamycin resistance gene (see col. 11, lines 3 and 20-22) may be used to detect plant cells that have been successfully transformed (see col 2., lines 26-29 and 33-35).

In imposing the rejection, the Office Action alleged that D'Halluin et al. could disclose the transformation of maize with a male sterile gene linked to a transgene of interest. As a result, the kanamycin resistance gene, or the bar gene, could

encode compounds that are therapeutic or prophylactic for the plant. In support of this argument, the Official Action cites to Example 5.

However, the therapeutic or prophylactic interest of the claimed invention arises in regards to a human or an animal, not to the plant. As a result, the kanamycin resistance gene, i.e. a gene conferring resistance to an antibiotic, cannot be regarded as encoding a therapeutic or prophylactic compound.

Indeed, the purpose of Example 5 is to produce male sterile maize. The successfully transformed embryos are selected by culturing with a selective medium which contains kanamycin (col. 20, lines 26-31). Therefore, D'Halluin et al. teach a method for selecting transformed plant cells, wherein the genome of a plant cell is transformed with a vector containing a male sterility gene and a gene encoding a selectable marker. D'Halluin et al. are not concerned with preventing transgene dissemination.

As a result, D'Halluin et al. do not teach nor suggest that the use of a barnase gene would prevent dissemination via pollen of a transgene encoding a therapeutic or prophylactic acid compound of human or animal origin genetically linked therewith. Therefore, the teaching of D'Halluin et al. would have given no suggestion to one skilled in the art as to how to carry out the claimed method.

As to Metz et al., Metz et al. are concerned with conferring pest resistance to plants. Metz et al. describe transformation of cytoplasmically sterile (i.e. acquired sterility) broccoli with a Bacillus thurigiensis (Bt) gene encoding an insecticidal crystal protein. Thus, Metz et al. are not concerned with the subject matter of the invention.

The Official Action asserts that one skilled in the art would have been motivated to modify the method of transformation taught by D'Halluin to transform plants with a gene encoding collagen. However, in D'Halluin et al., the gene of interest is the male sterility gene. Accordingly, the alleged modification of D'Halluin et al. would have lead to transforming plants with a construct carrying a gene encoding collagen and a selectable marker.

In light of this interpretation, and in order to benefit from the advantages of male sterility set forth by Metz et al., one skilled in the art would have had to transform a plant that was already male sterile with the construct carrying a gene encoding collagen and a selectable marker.

As a result, D'Halluin et al. in view of Metz et al. would not have motivated one skilled in the art to transform a plant, originally not male sterile, with a plasmid comprising both an artificial male sterility gene and a transgene encoding a

therapeutic or prophylactic compound of human of animal origin, so that transgene dissemination by pollen is prevented.

Thus, in view of the above, applicants believe that the proposed combination of publications fails to render obvious the claimed invention.

Claims 27-28 were rejected under 35 USC 103(a) as allegedly being unpatentable over Metz et al. in view of Vedel et al. Claims 31-32 are rejected under 35 USC 103(a) as allegedly being unpatentable over Metz et al. in view of Vedel et al. and further in view of each of Welter and Lenee et al. Applicants believe that the present amendment obviates this rejection.

Claims 27-28 and 31-32 have been canceled. Thus, applicants believe that these rejections have been rendered moot.

In view of the present amendment and foregoing remarks, therefore, applicants believe that the present application is in condition for allowance at the time of the next Official Action. Allowance and passage to issue on this basis is respectfully requested.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any

overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

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APPENDIX:

European Patent Application 0 344 029

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(54) **Plants with modified stamen cells.**

(57) A plant, the nuclear genome of which is transformed with a foreign DNA sequence encoding a product which selectively disrupts the metabolism, functioning and/or development of stamen cells of the plant. The foreign DNA sequence also optionally encodes a marker.

EP 0 344 029 A1

Description

PLANTS WITH MODIFIED STAMEN CELLS

This invention relates to a male-sterile plant and to its reproduction material (e.g., seeds), in which the cells are transformed so that a foreign DNA sequence is stably integrated into their nuclear genome. The foreign DNA sequence of this invention contains at least one first foreign DNA (hereinafter the "male-sterility DNA") that: 1) encodes a first RNA or protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, disturbs significantly the metabolism, functioning and/or development of the stamen cell; and 2) is in the same transcriptional unit as, and under the control of, a first promoter which is capable of directing expression of the male-sterility DNA selectively in stamen cells of the plant. In particular, this invention relates to such a nuclear male-sterile plant and its reproduction material, in which the forein DNA sequence of this invention is a foreign chimaeric DNA sequence that can also contain at least one second foreign DNA (the "marker DNA") that: 1) encodes a second RNA or protein or polypeptide which, when present at least in a specific tissue or specific cells of the plant, renders the entire plant easily separable from other plants that do not contain the second RNA, protein or polypeptide at least in the specific tissue or specific cells; 2) is in the same transcriptional unit as, and under the control of, a second promoter which is capable of directing expression of the marker DNA in at least the specific tissue or the specific cells of the plant; and 3) is in the same genetic locus of the nuclear genome of the cells of the plant as the male-sterility DNA.

This invention also relates to a foreign chimaeric DNA sequence that contains at least one male-sterility DNA under the control of the first promoter and that can also contain, adjacent to the male-sterility DNA, at least one marker DNA under the control of the second promoter.

This invention further relates to a vector that contains the foreign DNA sequence of this invention and is suitable for the transformation of plant cells, whereby the foreign DNA sequence is stably integrated into the nuclear genome of the cells.

This invention still further relates to cells of a plant and to plant cell cultures, the nuclear genomes of which are transformed with the foreing DNA sequence.

This invention yet further relates to a process for producing a nuclear male-sterile plant and its reproduction material and its cell cultures containing the foreign DNA sequence in which the male-sterility DNA: 1) is under the control of the first promoter and optionally in the same genetic locus as the marker DNA under the control of the second promoter; 2) is stably integrated into the nuclear genome of the plant's cells; and 3) can be expressed selectively in stamen cells of the plant in the form of the first RNA, protein or polypeptide.

The invention further relates to a process for producing hybrid seeds, which grow into hybrid plants, by crossing: 1) the male-sterile plant of this invention which includes, in its nuclear genome, the marker DNA, preferably encoding a protein conferring a resistance to a herbicide on the plant; and 2) a male-fertile plant without the marker DNA in its genome. This invention particularly relates to such a process for producing hybrid seeds on a commercial scale, preferably in a substantially random population, without the need for extensive hand-labor.

This invention still further relates to a tapetum-specific promoter from a plant genome. This promoter can be used as the first promoter in the foreign DNA sequence of this invention for transforming the plant to render it nuclear male-sterile.

Background of the Invention

Hybridization of plants is recognized as an important process for producing offspring having a combination of the desirable traits of the parent plants. The resulting hybrid offspring often have the ability to outperform the parents in different traits, such as in yield, adaptability to environmental changes, and disease resistance. This ability is called "heterosis" or "hybrid vigor". As a result, hybridization has been used extensively for improving major crops, such as corn, sugarbeet and sunflower. For a number of reasons, primarily related to the fact that most plants are capable of undergoing both self-pollination and cross-pollination, the controlled cross-pollination of plants without significant self-pollination, to produce a harvest of hybrid seeds, has been difficult to achieve on a commercial scale.

In nature, the vast majority of crop plants produce male and female reproductive organs on the same plant, usually in close proximity to one another in the same flower. This favors self-pollination. Some plants, however, are exceptions as a result of the particular morphology of their reproductive organs which favors cross-pollination. These plants produce hybrid offspring with improve vigor and adaptability. One such morphology in Cannabis ssp. (hemp) involves male and female reproduction organs on separate plants. Another such morphology in Zea mays (corn) involves male and female reproductive organs on different parts of the same plant. Another such morphology in Elaeis guineensis (oilpalm) involves male and fertile female gametes which become fertile at different times in the plant's development.

Some other plant species, such as Ananas comosus (pineapple), favor cross-pollination through the particular physiology of their reproductive organs. . Such plants have developed a so-called "self-incompatibility system" whereby the pollen of one plant is not able to fertilize the female gamete of the same plant or of another plant with the same genotype.

Some other plant species favor cross-pollination by naturally displaying the so-called genomic characteristic of "male sterility". By this characteristic, the plants' anthers degenerate before pollen, produced

by the anthers, reach maturity. See: "Male-Sterility in Higher Plants". M.L.H. Kaul, 1987, in: Monographs on Theoretical and Applied Genetics 10, Edit. Springer Verlag. Such a natural male-sterility characteristic is believed to result from a wide range of natural mutations, most often involving recessive deficiencies, and this characteristic can not easily be maintained in plant species that predominantly self-pollinate, since under natural conditions, no seeds will be produced.

There are four main types of male sterility observed in nature. All four types of male sterility are used in commercial breeding programs to ensure that there is cross-pollination to produce hybrid seed for crops such as corn, sugarbeet, oilseed rape and sunflower.

One type of male sterility is nuclear encoded and is believed to be inherited as a recessive allele. For breeding purposes, a recessive male-sterile parent plant is maintained by crossing it with a heterozygous male-fertile plant that also includes the recessive male-sterility allele, so that the offspring are 50% recessive male-sterile plants. The other 50% are male-fertile plants that have to be rogued out in outcrossing programs which can only be done efficiently if the recessive male-sterility allele is segregated together with a selectable or screenable marker. In US patent 4,727,219, a procedure is described for the use of recessive male sterility for the production of hybrid maize.

The second type of male sterility is nuclear encoded but inherited as a dominant allele. An advantage of dominant male sterile plants, as compared to recessive male sterile plants, is that the dominant male-sterile plants can be maintained through crossing with a male-fertile plant, to produce offspring that are 50% dominant male-sterile plants. The usefulness of this dominant nuclear male-sterile plant is, however, limited because its dominant male-sterility allele is in most cases not tightly linked (i.e., within the same genetic locus) to a selectable or screenable marker.

A third type of male sterility is cytoplasmatically encoded. In most cases, the cytoplasmic code is in the mitochondrial genome of the plant, and only in a few cases is the code in the chloroplast genome of the plant. The inheritance of cytoplasmatically encoded male sterility does not follow Mendelian rules but rather depends on cytoplasmic factors. The offspring obtained from crosses between cytoplasmic male-sterile plants and male-fertile plants all carry the cytoplasmic male-sterility gene and are therefore sterile. As a result, the offspring of plants of this type are only of commercial value if the economic product of the offspring is not for use as seed but rather for plants such as ornamentals and sugarbeet.

A fourth type of male sterility is the result of a combination of both nuclear encoded male sterility and cytoplasmatically encoded male sterility. The male sterility-inducing nuclear alleles are usually recessive, and only plants that contain the male-sterility cytoplasmic allele and that are homozygous for the male sterility-inducing nuclear allele are phenotypically male sterile. In this type of plant, corresponding dominant male fertility-inducing alleles or "restorers of fertility", produce a male-fertile phenotype. As a result, the male-sterile offspring of this type of plant can be made male-fertile by pollinating the male-sterile plants with pollen containing the restorers of fertility. As a result, the offspring of plants of this type are of commercial value where the economic product is seed, that is for plants such as corn, sorghum and sunflower.

Typically, hybrid seed production has been accomplished by the large scale planting of cytoplasmic male-sterile plants and male-fertile plants and by somehow (e.g., with a distinctive marker) preventing the resulting hybrid seeds from becoming mixed with non-hybrid seeds. According to U.S. patent no. 3,842,538, hybrid seeds are tediously separated from non-hybrid seeds on the basis of color. According to U.S. patent no. 4,351,130, the problem of separating hybrid seeds from non-hybrid seeds is avoided by using short male-sterile plants and tall male-fertile plants and then destroying the tall male-female plants after pollination. According to U.S. patents 4,658,085, 4,517,763 and 4,658,084, cytoplasmic male-sterile plants are provided with a herbicide tolerance absent from the male-fertile plants which are destroyed with the herbicide after pollination. According to U.S. patent no. 4,305,225, male-sterile rice plants are sprayed with a growth hormone (e.g., gibberellin) in order to cause fuller emergence of flower-bearing panicles from rice leaf sheaths, thereby increasing the ability of the flowers to receive pollen from male-fertile plants.

In all such processes for producing hybrid seeds from male-sterile plants, ways have been sought for simply and inexpensively obtaining on a commercial scale: 1) high hybrid seed production from each male-sterile plant; 2) a hybrid seed population that results almost exclusively from pollen of male-fertile plants and eggs of male-sterile plants and is substantially free of non-hybrid seeds from male-fertile plants; 3) easy production of both the male-sterile land male-fertile plants; and 4) the virtually complete removal or destruction of either the male-fertile plants after they have pollinated the male-sterile plants or the selective separation of non-hybrid seeds, produced by the male-fertile plants, from the hybrid seeds produced by the male-sterile plants.

Summary of the Invention

In accordance with this invention, a cell of a plant is provided, in which the nuclear genome is transformed with a foreign DNA sequence, preferably a foreign chimaeric DNA sequence, characterized by:

(a) a male-sterility DNA encoding a first RNA, protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, disturbs significantly the metabolism, functioning and/or development of the stamen cell; and

(b) a first promoter capable of directing expression of the male-sterility DNA selectively in stamen cells of the plant; the male-sterility DNA being in the same transcriptional unit as, and under the control of, the first promoter. The foreign DNA sequence in the nuclear genome of the transformed cell can also comprise, preferably in the same genetic locus as the male-sterility DNA:

(c) a marker DNA encoding a second RNA, protein or polypeptide which, when present at least in a specific tissue or specific cells of the plant, renders the plant easily separable from other plants which do not contain the second RNA, protein or polypeptide at least in the specific tissue or specific cells; and

(d) a second promoter capable of directing expression of the marker DNA at least in the specific tissue or specific cells; the marker DNA being in the same transcriptional unit as, and under the control of, the second promoter.

Also in accordance with this invention is provided a foreign chimaeric DNA sequence that comprises the male-sterility DNA and the first promoter and that can also comprise the marker DNA and the second promoter, as well as at least one additional DNA encoding a transit peptide capable of transporting the first protein or polypeptide or the second protein or polypeptide into a chloroplast or mitochondria of a plant cell in which the foreign chimaeric DNA sequence is expressed in its cytoplasm.

Further in accordance with this invention are provided: a male-sterile plant and a plant cell culture, each consisting of the transformed cells; a seed of the male-sterile plant; hybrid seeds and plants produced by crossing the male-sterile plant with a male-fertile plant; and a process for producing such hybrid seeds.

Still further in accordance withthis invention are provided tapetum-specific first promoters.

Description of the Invention

In accordance with this invention, a male-sterile plant is produced from a single cell of a plant by transforming the plant cell in a well known manner to stably insert, into the nuclear genome of the cell, the foreign DNA sequence of this invention. The foreign DNA sequence comprises at least one male-sterility DNA that is under the control of, and fused at its 5′ end to, the first promoter and is fused at its 3′ end to suitable transcription regulation signals (including a polyadenylation signal). Thereby, the first RNA, protein or polypeptide is produced or overproduced selectively in stamen cells of the plant so as to render the plant male-sterile. Preferably, the foreing DNA sequence also comprises at least one marker DNA that is under the control of, and is fused at its 5′ end to, the second promoter and is fused at its 3′ end to suitable transcription regulation signals (including a polyadenylation signal). The marker DNA is preferably in the same genetic locus as the male-sterility, whereby the second RNA, protein or polypeptide is produced in at least the specific tissue or specific cells of the plant so that the plant can be easily distingulshed and/or separated from other plants that do not contain the second RNA, protein or polypeptide in the specific tissue or specific cells. This guarantees, with a high degree of certainty, the joint segregation of both the male-sterility DNA and the marker DNA into offspring of the plant.

The cell of a plant (particularly a plant capable of being infected with Agrobacterium) is preferably transformed in accordance with this invention, using a vector that is a disarmed Ti-plasmid containing the foreign DNA sequence and carried by Agrobacterium. This transformation can be carried out using procedures described, for example, in European patent publications 0,116,718 and 0,270,822. Preferred Ti-plasmid vectors contain the foreign DNA sequence between the border sequence, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example, in European patent publication 0,223,247), pollen mediated transformation (as described, for example, in European patent publication 0,270,356, PCT publication WO85/01856, and European patent publication 0,275,069), in vitro protoplast transformation (as described, for example, in US patent 4,684,611), plant RNA virus-mediated transformation (as described, for example, in European patent publication 0,067,553, and US patent 4,407,956) and liposome-mediated transformation (as described, for example, in US patent 4,536,475).

Preferably, a nuclear male-sterile plant of this invention is provided by transforming a plant cell with a disarmed Ti-plasmid vector containing the foreign DNA sequence with both a male-sterility DNA under the control of a first promoter and a marker DNA under the control of a second promoter. The marker DNA can be upstream or downstream of the male-sterility DNA in the Ti-plasmid vector, but preferably, the two are adjacent to one another and are located between the border sequences or at least located to the left of the right border sequence of the Ti-plasmid vector, so that they are properly transferred together into the nuclear genome of the plant cell. However, if desired, the cell can initially be transformed with a foreign DNA sequence containing a male-sterility DNA and a first promoter and can subsequently be transformed with a marker DNA and a second promoter, inserted into the same genetic locus in the cell's nuclear genome as the male-sterility DNA. Suitable vectors for this purpose are the same as those discussed above for transforming cells with the foreign DNA sequence. The preferred vector is a disarmed Ti-plasmid vector.

The selection of the male-sterility DNA is not critical. A suitable male-sterility DNA can be selected and isolated in a well-known manner, so that it encodes the first RNA, protein or polypeptide which significantly disturbs the proper metabolism, functioning and/or development of any stamen cell in which the male-sterility DNA is expressed, preferably leading thereby to the death of any such stamen cell. Preferred examples of male-sterility DNAs encode: RNases such as RNase T1 (which degrades RNA molecules by hydrolyzing the bond after any guanine residue) and Barnase; DNases such as an endonuclease (e.g., EcoRI); or proteases such as a papain (e.g., papain zymogen and papain active protein).

Other examples of male-sterility DNAs encode enzymes which catalyse the synthesis of phytohormones, such as: isopentenyl transferase which is an enzyme that catalyzes the first step in cytokinin biosynthesis and is encoded by gene 4 of Agrobacterium T-DNA; and the enzymes involved in the synthesis of auxin and encoded by gene 1 and gene 2 of Agrobacterium T-DNA. Yet other examples of male-sterility DNAs encode:

glucanases; lipases such as phospholipase A_2 (Verheij et al (1981) Rev. Biochem. Pharmacol. 91, 92-203); lipid peroxidases; or plant cell wall inhibitors. Still other examples of male-sterility DNAs encode proteins toxic to plants cells, such as a bacterial toxin (e.g., the B-fragment of diphtheria toxin or botulin).

Still another example of a male-sterility DNA is an antisense DNA which encodes a strand of DNA complementary to a strand of DNA that is naturally transcribed in the plant's stamen cells under the control of an endogenous promoter as described, for example, in European patent publication 0,223,399. Such an antisense DNA can be transcribed into an RNA sequence capable of binding to the coding and/or non-coding portion of an RNA, naturally produced in the stamen cell, so as to inhibit the translation of the naturally produced RNA. An example of such an antisense DNA is the antisense DNA of the TA29 gene (described in Example 2) which is naturally expressed, under the control of the TA29 promoter, in tapetum cells of the anthers of plants.

A further example of a male-sterility DNA encodes a specific RNA enzyme (i.e., a so-called "ribozyme"), capable of highly specific cleavage against a given target sequence, as described by Haseloff and Gerlach (1988) Nature 334, 585-591. Such a ribozyme is, for example, the ribozyme targeted against the RNA encoded by the TA29 gene.

Still other examples of male-sterility DNAs encode products which can render the stamen cells susceptible to specific diseases, such as fungus infections. Such a male-sterility DNA can be used in a plant wherein all other cells, in which the male-sterility DNA is not expressed, are resistant to the specific disease.

By "foreign" with regard to the foreign DNA sequence of this invention is meant that the foreign DNA sequence contains a foreign male-sterility DNA and/or a foreign first promoter. By "foreign" with regard to a DNA, such as a male-sterility DNA and a first promoter, as well as marker DNA, a second promoter and any other DNA in the foreign DNA sequence, is meant that such a DNA is not in the same genomic environment in a plant cell, transformed with such a DNA in accordance with this invention, as is such a DNA when it is naturally found in the cell of the plant, bacteria, animal, fungus, virus, or the like, from which such a DNA originates. This means, for example, that a foreign male-sterility DNA or marker DNA can be: 1) a nuclear DNA in a plant of origin; 2) endogenous to the transformed plant cell (i.e., from a plant or origin with the same genotype as the plant being transformed); and 3) within the same transcriptional unit as its own endogenous promoter and 3′ end transcription regulation signals (from the plant of origin) in the foreign DNA sequence of this invention in the transformed plant cell; but 4) inserted in a different place in the nuclear genome of the transformed plant cell than it was in the plant of origin so that it is not surrounded in the transformed plant cell by the genes which surrounded it naturally in the plant of origin. A foreign male-sterility or marker DNA can also, for example, be: 1) a nuclear DNA in a plant of origin; and 2) endogenous to the transformed plant cell; but 3) in the same transcriptional unit as a different (i.e., not its own) endogenous promotor and/or 3′ end transcription regulation signals in a foreign chimaeric DNA sequence of this invention in a transformed plant cell. A foreign male-sterility or marker DNA can also, for example, be: 1) a nuclear DNA in a plant of origin; and 2) endogenous to the transformed plant cell; but 3) in the same transcriptional unit as a heterologous promotor and/or 3′ end transcription regulation signals in a foreign chimaeric DNA sequence of this invention in a transformed plant cell. A foreign male-sterility or marker DNA can also, for example, be heterologous to the transformed plant cell and in the same transcriptional unit as an endogenous promotor and/or 3′ transcription regulation signals (e.g., from the nuclear genome of a plant with the same genotype as the plant being transformed) in a foreign chimaeric DNA sequence of this invention in a transformed plant cell. An example of a foreign male-sterility DNA could come from the nuclear genome of a plant with the same genotype as the plant being transformed and encode a catalytic enzyme, such as a protease or ribonuclease, that is endogenous to stamen cells of the plant being transformed, so that the enzyme is overproduced in transformed stamen cells in order to disturb significantly their metabolism, functioning and/or development. Preferably, the male-sterility DNA and the marker DNA are each heterologous to the plant cell being transformed.

By "heterologous" with regard to a DNA, such as a male-sterility DNA, a first promoter, a marker DNA, a second promoter and any other DNA in the foreign DNA sequence, is meant that such a DNA is not naturally found in the nuclear genome cells of a plant with the same genotype as the plant being transformed. Examples of heterologous DNAs include chloroplast and mitochondrial DNAs obtained from a plant with the same genotype as the plant being transformed, but preferred examples are chloroplast, mitochondrial, and nuclear DNAs from plants having a different genotype than the plant being transformed, DNAs from animal and bacterial genomes, and chromosomal and plasmidial DNAs from fungal and viral genomes.

By "chimaeric" with regard to the foreign DNA sequence of this invention is meant that at least one of its male-sterility DNAs: 1) is not naturally found under the control of its first promoter for the one male-sterility DNA; and/or 2) is not naturally found in the same genetic locus as at least one of its marker DNAs. Examples of foreign chimaeric DNA sequences of this invention comprise: a male-sterility DNA of bacterial origin under the control of a first promoter of plant origin; and a male-sterility DNA of plant origin under the control of a first promoter of plant origin and in the same genetic locus as a marker DNA of bacterial origin.

So that the male-sterility DNA is expressed selectively in stamen cells of a plant, it is preferred that the first promoter, which controls the male-sterility DNA in the foreign DNA sequence, be a promoter capable of directing gene expression selectively in stamen cells of the plant. (By "stamen" is meant the organ of the flower that produces the male gamete and that includes an anther and a filament). Such a stamen-specific promoter can be an endogenous promoter or an exogenous promoter and can be from the nuclear genome or from the mitochondrial or chloroplast genome of a plant cell. In any event, the first promoter is foreign to the

nuclear genome of the plant cell, being transformed. Preferably, the first promoter causes the male-sterility DNA to be expressed only in anther, pollen or filament cells, especially in tapetum or anther epidermal cells. The first promoter can be selected and isolated in a well known manner from the species of plant, to be rendered male-sterile, so that the first promoter directs expression of the male-sterility DNA selectively in stamen cells so as to kill or disable the stamen and render the plant incapable of producing fertile male gametes. The first promoter is preferably also selected and isolated so that it is effective to prevent expression of the male-sterility DNA in other parts of the plant that are not involved in the production of fertile pollen, especially in female organs of the plant. For example, a suitable endogenous stamen-specific first promoter can be identified and isolated in a plant, to be made male-sterile, by:

1. searching for an mRNA which is only present in the plant during the development of its stamen, preferably its anthers, pollen or filament;
2. isolating this stamen-specific mRNA;
3. preparing a cDNA from this stamen-specific mRNA;
4. using this cDNA as a probe to identify the regions in the plant genome which contain DNA coding for the stamen-specific mRNA; and then
5. identifying the portion of the plant genome that is upstream (i.e., 5') from the DNA coding for the stamen-specific mRNA and that contains the promoter of this DNA.

Examples of such first promoters are the TA29 promoter, the TA26 promoter and the TA13 promoter, hereinafter described in the Examples, which have been isolated from tobacco and are tapetum-specific promoters. Another tapetum-specific first promoter from another plant species can be isolated from its genome, using the TA29, TA26 or TA13 gene as a probe as in step 4, above. Under hybridizing conditions, such a probe will hybridize to DNA coding for a tapetum-specific mRNA in a mixture of DNA sequences from the genome of the other plant species (Maniatis et al (1982) Molecular Cloning. A Laboratory Manual. Ed. Cold Spring Harbor Laboratory). Thereafter, as in step 5 above, the other tapetum-specific first promoter can be identified.

If more than one male-sterility DNA is present in the foreign DNA sequence of this invention, all the male-sterility DNAs can be under the control of a single first promoter, but preferably, each male-sterility DNA is under the control of its own separate first promoter. Where a plurality of male-sterility DNAs are present in the foreign DNA sequence, the male-sterility DNA also can encode the same or different first RNA(s), polypeptide(s) and protein(s). For example, when the male-sterility DNA encodes an RNase such as RNase T1, it preferred that at least 3, particularly 4 to 6, copies of the male-sterility DNA and its first promoter be provided in the foreign DNA sequence. In any event, all the male-sterility DNA(s) and their first promoter(s) are preferably adjacent to one another in the foreign DNA sequence land in any vector used to transform plant cells with the foreign DNA sequence.

The selection of the marker DNA also is not critical. A suitable marker DNA can be selected and isolated in a well known manner, so that it encodes a second RNA, protein or polypeptide that allows plants, expressing the marker DNA, to be easily distinguished and separated from plants not expressing the second RNA, protein or polypeptide . Examples of marker DNAs encode proteins that can provide a distingulshable color to plant cells, such as the A1 gene encoding dihydroquercetin-4-reductase (Meyer et al (1987) Nature 330, 677-678) and the glucoronidase gene (Jefferson et al (1988) Proc. Natl. Acad. Sci. USA ("PNAS") 83, 8447), or that provide a specific morphological characteristic to the plant such as dwarf growth or a different shape of the leaves. Other examples of marker DNAs confer on plants: stress tolerance, such as is provided by the gene encoding superoxide dismutase as described in European patent application 88/402222.9; disease or pest resistance such as is provided by a gene encoding a Bacillus thuringiensis endotoxin conferring insect resistance as described in European patent application 86/300291.1 or a gene encoding a bacterial peptide that confers a bacterial resistance as described in European patent application 88/401673.4

Preferred marker DNAs encode second proteins or polypeptides inhibiting or neutralizing the action of herbicides such as: the sfr gene and the sfrv gene encoding enzymes conferring resistance to glutamine synthetase inhibitors such as Biolaphos and phosphinotricine as described in European patent application 87/400,544.0; genes encoding modified target enzymes for certain herbicides that have a lower affinity for the herbicides than naturally produced endogenous enzymes, such as a modified glutamine synthetase as target for phosphinotricine as described in European patent publication 0,240,792 and a modified 5-enolpyruvylshikimate-3 phosphate synthase as a target for glyphosate as described in European patent publication 0,218,571.

The second promoter, which controls the marker DNA, can also be selected and isolated in a well known manner so that the marker DNA is expressed either selectively in one or more specific tissues or specific cells or constitutively in the entire plant, as desired depending on the nature of the second RNA, protein or polypeptide encoded by the marker DNA. For example, if the marker DNA encodes an herbicide resistance, it may be useful to have the marker DNA expressed in all cells of the plant, using a strong constitutive second promoter such as a 35S promoter (Odell et al (1985) Nature 313, 810-812), a 35S'3 promoter (Hull and Howell (1987) Virology 86, 482-493), the promoter of the nopaline synthetase gene ("PNOS") of the Ti-plasmid (Herrera Estrella (1983) Nature 303, 209-213) or the promoter of the octopine synthase gene ("POCS" [De Greve et al (1982) J. Mol. Appl. Genet. 1 (6), 499-511]). If the marker DNA encodes a protein conferring disease resistance, it may be useful to have the marker DNA selectively expressed in wound tissue by using, for example, a TR promoter such as the TR1' or TR2' promoter of the Ti-plasmid (Velten et al (1984) EMBO J. 3, 2723-2730). If the marker DNA encodes a herbicide resistance, it may be useful to have the marker DNA

selectively expressed in green tissue by using, for example, the promoter of the gene encoding the small subunit of Rubisco ((European patent application 87/400,544.0). If the marker DNA encodes a pigment, it may be useful to have the marker DNA expressed in specific cells, such as petal cells, leaf cells or seed cells, preferably in the outside layer of the seed coat.

One can identify and isolate in a well known manner a tissue-specific second promoter for a plant to be rendered male-sterile and easily distinguishable from non-transformed plants by:

1. searching for an mRNA which is only present in the plant during the development of a certain tissue, such as its petals, leaves or seeds;
2. isolating this tissue-specific mRNA;
3. preparing a cDNA from this tissue-specific mRNA;
4. using this cDNA as a probe to identify the regions in the plant genome which contain DNA coding for the tissue-specific mRNA; and then
5. identifying the portion of the plant genome that is upstream from the DNA coding for the tissue-specific mRNA and that contains the promoter for said DNA.

If more than one marker DNA is present in the foreign DNA sequence of this invention, all the marker DNAs can be under the control of a single second promoter, but preferably, each marker DNA is under the control of its own separate second promoter. More preferably, each marker DNA is under the control of its own second promoter and encodes a different second RNA, protein or polypeptide, providing different distinguishable characteristics to a transformed plant. In any event, the marker DNA(s) and second promoter(s) should be adjacent to each other and to the one or more male-sterility DNAs contained in the foreign DNA sequence of this invention and in any vector used to transform plant cells with the foreign DNA sequence.

It is generally preferred that the first RNA, protein or polypeptide, encoded by the male-sterility DNA, interfere significantly with the stamen cells' metabolism, functioning and/or development by acting in the cytoplasm or the nucleus of the stamen cells. However, when it is desired to have the first protein or polypeptide and/or of the second protein or polypeptide transported from the cytoplasm into chloroplasts or mitochondria of the cells of transformed plants, the foreign DNA sequence can further include an additional foreign DNA encoding a transit peptide. The additional DNA is between the male-sterility DNA and the first promoter is the first protein or polypeptide is to be so-transported and is between the marker DNA and the second promoter if the second protein or polypeptide is to be so-transported. By "transit peptide" is meant a polypeptide fragment which is normally associated with a chloroplast or mitochondrial protein or subunit of the protein and is produced in a cell as a precursor protein encoded by the nuclear DNA of the cell. The transit peptide is responsible for the translocation process of the nuclear-encoded chloroplast or mitochondrial protein or subunit into the chloroplast or the mitochondrial, and during such a process, the transit peptide is separated or proteolytically removed form the chloroplast or mitochondrial protein or subunit. One or more of such additional DNA's can be provided in the foreign DNA sequence of this invention for transporting one or more first or second proteins or polypeptides as generally described in European patent applications 85/402,596.2 and 88/402,222.9 and in: Van den Broeck et al (1985) Nature 313, 358-363; Schatz (1987) Eur. J. of Bioch. 165, 1-6; and Boutry et al (1987) Nature 328, 340-342. An example of a suitable transit peptide for transport into chloroplasts is the transit peptide of the small subunit of the enzyme RUBP carboxylase (European patent application 85/402,596.2) and an example of a transit peptide for transport into mitochondria is the transit peptide of the enzyme Mn-superoxide dismutase (see Example 16).

In the foreign DNA sequence of this invention, 3' transcription regulation signals can be selected among those which are capable of enabling correct transcription termination and polyadenylation of mRNA in plant cells. The transcription regulation signals can be the natural ones of the gene to be transcribed but can also be foreign or heterologous. Examples of heterologous transcription regulation signals are those of the octopine synthase gene (Gielen et al (1984) EMBO J. 3,, 835-845) and the T-DNA gene 7 (Velten and Schell (1985) Nucleic Acids Research ("NAR") 13, 6981-6998).

Also in accordance with this invention, plant cell cultures, such as anther cell cultures, containing the foreign DNA sequence of this invention in which the first promoter effects expression of the male-sterility DNA at a given stage of pollen development, more especially after meiosis, can be used to regenerate homozygous dominant male-sterile plants ("Efficient isolation of microspores and the production of microspore-derived embryos from Brassica napus", E.B. Swanson, M.P. Coumans, S.C. Wu, T.L. Barby and W.D. Beversdorf, Plant Cell Reports (1987) 6: 94-97).

Further in accordance with this invention, processes are provided for producing hybrid seeds which can be grown into hybrid plants. One process involves crossing a nuclear male-sterile plant including at least one marker DNA with a male-fertile plant without the marker DNA. Both male-sterile and male-fertile plants are planted in separate rows near to each other. Another process involves crossing a nuclear male-sterile plant including at least two different marker DNAs with a male-fertile plant including, in common, only one of the two different marker DNAs in a homozygous form. Both male-sterile and male-fertile parent plants can be grown in a substantially random population, increasing the chances of cross-pollination, without the need for precise planting patterns. The male-fertile parent plant can thereafter be easily removed from the population, using the distinctive trait encoded by the non-common marker DNA which is not possessed by the male-fertile parent plant. Preferably in this process, the non-common marker DNA in the male-sterile plant is under the control of a constitutive promoter and encodes a protein or polypeptide that renders the male-sterile plant resistant to a particular herbicide. The male-fertile plant can then be destroyed after cross-pollination, using the particular

herbicide.

Plants, transformed with the male-sterility DNA, preferably with both the male-sterility DNA and the marker DNA encoding herbicide-resistance, stably integrated and transmissible throughout generations as dominant alleles in accordance with this invention, are alternatives to, and provide several advantages over, presently used cytoplasmic male-sterility systems for breeding and producing hybrid crops. Such advantages include:

1. For cross-pollinating crops, the breeding strategy is much simplified, because it is not necessary to introduce a restorer gene into the male-fertile parent line of the cross that will produce the commercially sold hybrid seed. Indeed, a heterozygous nuclear male-sterile parent line crossed with another male-sterile parent line for commercial seed production will produce 50% male-sterile hybrid offspring and 50% male-fertile hybrid offspring, as a result of which the commercial crop will produce enough pollen to guarantee full seed set and therefore normal yield. Examples for such crops are corn and oilseed rape.

2. For crops for which the seeds do not represent the economic harvest, the breeding strategy is also much simplified without the need of a restorer gene expressed in the male-fertile parent line. Indeed, for these crops it does not matter that 50% of the commercially sold hybrid seeds are male-sterile. Examples for these crops are sugarbeet and alfalfa.

3. The system allows production of nuclear male-sterile lines and maintainer lines from existing inbred lines in one operation, eliminating the need for backcrossing. This reduces the time lag between conception and commercialization of a hybrid by at least 6 to 8 generations. An example of a typical strategy for producing hybrid plants using as parent plant the plants having inserted and expressing the male-sterility DNA may consist of the following steps:

1) making test hybrids by hand, by crossing inbred lines, and testing for combining ability and selected characteristics (2 years).

2) making one parent line of each of the selected hybrids nuclear male-sterile by the process which is the object of this invention (1 year).

3) multiplying the nuclear male sterile parent plant obtained from said process, hereinafter called "A^S", and its maintainer line, hereinafter called "A", and the pollinating male-fertile parent plant, hereinafter called "B", of the future commercial crop (3 years). During the same period, introducing the selected hybrids in official yield trials (3 years).

4) producing and selling the approved hybrid seed (1 year).

4. Combined with a marker DNA encoding herbicide-resistance, such a nuclear male-sterility system allows production of 2-, 3- and 4- way hybrids in any combination required. It is believed to be sufficient to introduce the male-sterility DNA and adjacent thereto the marker DNA into the nuclear genome of one plant which will be used as one of the grandparent breeding lines for obtaining 2- or 3-way hybrids, and into the nuclear genome of two plants which will be used as the two grandparent lines for 4-way hybrids. Each breeding line can be maintained by the following two crosses given here by way of example, and whereby "SH" stands for the dominant alleles respectively of male-sterility (S) and herbicide resistance (H), and sh stands forthe recessive alleles respectively of male fertility (s) and herbicide sensitivity (h):

a. SH/sh x sh/sh gives 50% SH and 50% sh offspring, and after spraying with the herbicide to which H confers resistance, 100% sterile seedlings are obtained.

b. sh/sh x sh/sh gives 100% fertile offspring.

5. It provides a protection for the owner of the marker DNA that has been integrated into the male-sterility system by making it more difficult for competitors to breed the marker DNA into their own breeding lines.

For illustrative purposes, two crop breeding schemes in accordance with this invention are given as follows:

Scheme 1: Breeding a plant containing adjacent male-sterility DNA and marker DNA encoding herbicide-resistance

1A) maintaining the male-sterility line A^S:
line $A^{SH/sh}$ x line $A^{sh/sh}$
giving
50% $A^{SH/sh}$ (phenotype: male-sterile, herbicide-resistant)
50% $A^{sh/sh}$ (phenotype: male-fertile, herbicide-susceptible)

1B) producing the hybrid seed crop:

a) planting seeds of $B^{sh/sh}$ (male plants) and the seeds obtained by the cross 1A) consisting of $A^{SH/sh}$ and $A^{sh/sh}$ ("female" plants) in separate rows.

b) eliminating the genotype $A^{sh/sh}$ by spraying the female rows with the herbicide.

c) cross-pollination occurring:
$A^{SH/sh} \times B^{sh/sh} \times B^{sh/sh}$
giving in the female rows:
50% $AB^{SH/sh}$ (phenotype: hybrid, male-sterile, herbicide-resistant)
50% $AB^{sh/sh}$ (phenotype: hybrid, male-fertile, herbicide-sensitive)

and in the male rows: 100% $B^{sh/sh}$.

d) eliminating the genotype $B^{sh/sh}$ occurring in the male rows by spraying with the herbicide or by mechanical means.

e) harvesting the hybrid seeds of the female rows wherein the cross-pollination of c) occurred. This is the commercially sold seed.

Scheme 2: Breeding a plant containing adjacent male-sterility DNA and two marker DNAs, each encoding a different herbicide-resistance (H1 and H2).

2A) maintaining the male-sterile line A^S:
A^S:$A^{SH1H2/sh1h2}$ x $A^{sh1h2/sh1h2}$
giving
50% $A^{SH1H2/sh1h2}$ (phenotype: male-sterile, resistant to both herbicides).
50% $A^{sh1h2/sh1h2}$ (phenotype: male-fertile, susceptible to both herbicides).

2B) maintaining pollination line B:
$B^{sh1H2/sh1H2}$ X $B^{sh1H2/sh1H2}$
giving
100% $B^{sh1H2/sh1H2}$ (phenotype: male-fertile, susceptible to herbicide 1 and resistant to herbicide 2).

2C) producing the hybrid seed crop:

a) planting the seeds obtained from 2A) and the seeds obtained from 2B) at random.

b) eliminating the genotype $A^{sh1h2/sh1h2}$ by spraying the field with herbicide 2.

c) cross-pollination occurring:
$A^{SH1H2/sh1h2}$ x $B^{sh1H2/sh1H2}$
giving
50% $AB^{SH1H2/sh1H2}$
50% $AB^{sh1h2/sh1H2}$
and
self-pollination occurring:
$B^{sh1H2/sh1H2}$ x $B^{sh1H2/sh1H2}$
giving
100% $B^{sh1H2/sh1H2}$

d) eliminating plants with genotype $B^{sh1H2/sh1H2}$ obtained from the parent line B, for which self-pollination occurred, by spraying the field with herbicide 1.

e) harvesting hybrid seeds of the remaining plants $A^{SH1H2/sh1H2}$ obtained by the cross-pollination of c).

The following Examples illustrate the invention. The figures referred to in the Examples are as follows:

Fig. 1. shows restriction maps of TA29 cDNA and its ClaI fragment in pTA29S3 of Example 1.

Fig. 2 shows the cDNA sequence of the PstI fragment of the TA29 gene of Example 2.

Fig. 3A shows the DNA sequence and amino acid sequence of the TA29 gene, from its ClaI site to its HindIII site. Above the sequences, the important restriction sites are indicated, and under the sequences is the amino acid sequence encoded by the ORF. Also indicated are:
- from nucleotide ("nt") 1446 to 1452: TATA box (asterisks),
- at nt 1477: transcription initiation site of TA29 mRNA (asterisk),
- from nt 1514 to 1537: the 3′ to 5′ sequence of a synthetic oligomer as described in Example 2, and
- from nt 1940 to 2296 (between arrows); the aligned sequence of TA29 cDNA.

Fig. 3B shows the alignment of the TA13 cDNA (top line) and the TA29 cDNA (bottom line); as discussed in Example 4. Homologous nucleotides are indicated by vertical lines.

Fig. 3C shows the sequence of the TA26 cDNA, as discussed in Example 4; the ORF is underlined.

4A shows schematically the construction of the vector pMB2 of Example 3.

Fig. 4B shows a map of the vector pMB3 of Example 3.

Fig. 5 shows a map of the vector pTTM3 of Example 5.

Fig. 6 shows a map of the vector pTTM4 of Example 7.

Fig. 7A shows a map of the vector pTTM6 of Example 9.

Fig. 7B shows a map of the vector PTTM6A$^-$ of Example 11.

Fig. 8 shows a map of the vector pTTM8 of Example 12.

Fig. 9A shows a map of the vector pTVEP1 of Example 14.

Fig. 9B shows a map of the vector pTVEP2 of Example 14.

Fig. 10A shows a map of the vector pTVEP63 of Example 16.

Fig. 10B shows a map of the vector pTVEP62 of Example 16.

Fig. 11 shows a photograph of flowers of normal tobacco plants compared with flowers of tobacco plants transformed with the male-sterility DNA of Example 9.

Fig.12 shows a photograph of a transverse cutting of the anther of a normal tobacco plant compared with the anther of a tobacco plant transformed with the male-sterility DNA of Example 9 (enhancement: x 250).

Unless otherwise stated in the Examples, all procedures for making and manipulating recombinant DNA were carried out by the standardized procedures described in Maniatis et al, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory (1982). The following plasmids and vectors, used in the Examples, have been deposited in the Deutsche Sammlung Für Mikroorganismen und Zellculturen ("DSM"), Mascheroder Weg 1B, D-330 Braunschweig, Federal Republic of Germany under the provisions of the Budapest Treaty:

Plasmid or vector	DSM Accession No.	Date
pMB3	4470	21 Mar. 1988
pGSC1600	4467	21 Mar. 1988
pGCC1700	4469	21 Mar. 1988
pGV2260	2799	Dec. 1983
pGSC1701A	4286	22 Oct. 1987
pTTM4	4471	21 Mar. 1988
pMAC5-8	4566	25 April 1988
pTTM6	4468	21 Mar. 1988

Example 1 - Subcloning of an anther-specific gene (the "TA29 gene")

From Professor Robert Goldberg of the University of California, Los Angeles (UCLA) were obtained: a Nicotiana tabacum anther-specific cDNA ("TA29 cDNA") cloned as a PstI fragment in pBR329 (Covarrubias and Bolivar (1982) Gene 17, 79) by GC tailing; and the corresponding genomic clone ("lambda TA29") that was isolated from a N. tabacum "Samsun" genomic library using TA29 cDNA as a probe and that was inserted in the EcoRI site of the lambda phage vector cH32 (Loenen and Blattner (1983) Gene 26, 171). The TA29 cDNA was 365 base pairs long (±0.4 kb) and hybridized to a tapetum-specific mRNA of 1,100 nucleotides which accounts for 0.24% of the poly A^+ mRNA from anthers of the N. tabacum. As shown in Fig. 1, lambda TA29 contains two EcoRI fragments, the total insert measuring 13.2 kb.

An internal 7.5 kb ClaI fragment as shown in Fig. 1, containing the TA29 gene, was subcloned from lambda TA29 in pLK31 (Botterman and Zabeau (1987) DNA 6, 6) which produced a plasmid names "pTA29S3". Nitrocellulose bound fragments of lambda TA29, digested with the combination of EcoRI/ClaI/HindIII/HindIII-EcoRI and the combination of ClaI-EcoRI and hybridized against TA29 cDNA, indicated the presence of sequences homologous to TA29 cDNA.

Example 2 - Nucleotide sequence determination of TA29 cDNA and its homologous sequence from pTA29S3; mapping of TA29 gene and its promoter.

The PstI insert of TA29 cDNA in pBR329 was completely sequenced (Maxam and Gilbert (1977) Proc. Natl. Acad. Sci. USA ("PNAS") 74, 560). The cDNA sequence is shown in Fig. 2. It reveals the presence of one open reading frame over the entire cDNA sequence (as indicated).

Then, the sequence of the ClaI insert in pTA29S3 was determined from the ClaI site to the HindIII site (3261 base pairs apart). Comparison of the TA29 cDNA sequence and the pTA29S3 sequence revealed the presence of a sequence in the pTA29S3 which was completely homologous with the TA29 cDNA sequence.

Figure 3 shows the sequence of the TA29 gene in pTA29S3. The sequence in pTA29S3 that is identical to the TA29 cDNA sequence is between the arrows in Fig. 3. A putative open reading frame is revealed by the corresponding amino acid sequence in Fig. 3. This indicates that the TA29 gene encodes a protein of 321 amino acid residues and that there are no introns present in the coding region. The length of the open reading frame of 964 (+ leader) nucleotides matches the size of a transcript present in tobacco anther mRNA prepared from anthers isolated from young (12-20 mm long) tobacco flower buds and absent in the mRNA isolated from leaf and older flowers (when the buds are opened and petals have appeared). The size of this mRNA is approximately 1100 nucleotides.

There are two ATG codons, one at nucleotide ("nt") 1527 and the other at nt 1560, which could serve as initiation codon for the open reading frame, 33 nucleotides apart. There is a consensus sequence TATA at nt 1446 present 81 nucleotides 5′ upstream of the first ATG codon (indicated by asterisks in Fig. 3). To confirm that this "TATA" box is part of the promoter of the TA29 gene, the 5′ end of the TA29 mRNA was determined. This was done by primer extension (Mc Knight et al (1981) Cell 25, 385). For this purpose, an oligomer of 24 nucleotides, having the sequence: 5′ GGA GCT ACC ATT TTA CGT AAT TTC 3′, was used as it is complementary to the TA29 gene from nt 1514 to nt 1537 as shown in Fig. 3.

This oligonucleotide was ^{32}P labeled by kination at the 5′ end. After being hybridized with anther mRNA, the oligonucleotide was extended by reverse transcriptase. The resulting extended oligonucleotide was analyzed on a sequencing gel, next to a sequencing ladder, to determine its exact size. The fragment was shown to be 61 nucleotides long. This indicates that transcription initiation of the TA29 mRNA occurred at nt 1477 (indicated by asterisk in Fig. 3). Hence, the TA29 gene has a TATA box located 31 nucleotides upstream of the transcription initiation site. The mRNA contains a 51 nucleotide-long leader sequence from nt 1477 to nt 1527,

a coding region of 964 nucleotides from nt 1527 to nt 2491, and a 3′ non coding region of approximately 100 nucleotides from nt 2492 to nt 2590. As is the case in approximately 92% of presently characterized plant genes (Joshin (1987) Nucleic Acids Research ("NAR") 15 (16), 6643), it is believed that the first AUG codon of the mRNA is used to initiate translation. The TA29 promoter thus appears to be located between the ClaI restriction site and nt 1477.

Example 3 - Construction of a promoter cassette ("PTA29") derived from the TA29 gene

To construct chimaeric DNA sequences containing the 5′ regulatory sequences, including the promoter, of the TA29 gene in the same transcriptional unit as, and controlling, a first heterologous male-sterility DNA, a cassette was constructed as shown in Fig. 4 by subcloning a 2.5 kb ClaI/AccI fragment from pTA29S3 into the polylinker AccI site of pMAC 5-8 (European patent application 87/402348.4). This produced a vector named "pMB2", shown in Fig. 4, which could be used to isolate single strand DNA for use in site directed mutagenesis.

Then, the sequence surrounding the first ATG codon AAAATGGTA was modified to ACCATGGTA by substituting two adenine residues for cytosine residues. This mutation created the sequence CCATGG which is the recognition site for the restriction enzyme NcoI. This site directed mutagenesis in pMB2 was performed using a synthetic oligonucleotide of 24 nucleotides with the following sequence:

3′GTT TAA TCG ATG GTA CCA TCG AGG 5′

The resulting plasmid, containing the newly created NcoI site, was named "pMB3" and is shown in Fig. 4 bis. The precise nucleotide sequence spanning the NcoI site was determined in order to confirm that it only differed from the 5′ sequence of the TA29 gene by the AA -- CC substitution, creating the NcoI site. The 1507 nucleotide long fragment ClaI -- NcoI was named "PTA29".

Example 4 - Identification of cDNA clones obtained from other stamen-specific mRNAs

To demonstrate that other anther-specific mRNAs could be identified and then used to isolate cDNA clones with analogous properties to the TA29 gene, two other N. tabacum anther-specific cDNAs ("TA13 cDNA" and "TA26 cDNA") were obtained from Professor Goldberg of UCLA.

TA13 cDNA is a clone of 1100 bp which hybridized to two mRNA species of about 1100 and 1200 nucleotides, respectively, which are specific for tapetum cells and are abundant at a very early stage of anther development. TA13 cDNA was sequenced, using the procedure of Example 2, and then compared with the sequence of TA29 cDNA as shown in Fig. 3B. This sequence comparison reveals that TA13 cDNA and TA29 cDNA share 92% homology, and the ORF is very rich in glycine content.

TA26 cDNA was cloned as a PstI insert into pBR329 by poly-G/C tailing. It is a clone of 519 bp which hybridized to one tobacco mRNA species of 580 nucleotides, which mRNA is specific for tapetum cells and abundant at a certain stage of anther development. The entire TA26 cDNA was sequenced, using the procedure of Example 2, and when compared with the sequence of TA29 cDNA, revealed no homology. The sequence of TA26 cDNA is given in Fig. 3C.

Example 5 - Construction of a chimaeric DNA sequence of PTA29 and a glucuronidase gene

A plasmid named "pTTM3", shown in Fig. 5, was constructed by assembling the following well known DNA fragments:

1. a vector fragment, including T-DNA border sequences, derived from pGSC1600;
2. a chimaeric sequence containing the promoter cassette PTA29 from Example 3, fused in frame with a pMB3 NcoI/EcoRI fragment containing an E. coli gene encoding beta-glucuronidase ("GUS" [Jefferson et al (1986) PNAS 83, 8447; Jefferson et al (1987) EMBO J. 6, 3901]) and the 3′ end signals of an octopine-synthase gene ("OCS" [Dhaese et al (1983) EMBO J. 2, 419]);
3. a chimaeric sequence containing an Arabidopsis SSU promoter ("PSSU") or "PSSUARA), a herbicide resistance gene sfr (European patent application 87/400,544.0) and the 3′ end signals of a T-DNA gene 7 (Velten and Schell (1985) NAR 13, 6981); and
4. A chimaeric sequence containing the EcoRI/SacI fragment from pGSFR401 which contains a nopaline-synthase promoter ("PNOS"), a neo gene encoding kanamycin resistance and the 3′ end signals of an octopine synthase gene (European patent application 87/400,544.0, wherein pGSFR401 is called "pGSR4"). pTTM3 is a T-DNA vector containing, within the T-DNA border sequences, two chimaeric sequences: PSSU-sfr in which the sfr is a marker DNA (European patent application 87/400,544.0) under the control of PSSU as a second promoter; and PTA29-GUS in which GUS is a reporter gene whose expression in plants and plant cells under the control of the TA29 promoter can easily be localized and quantified.

Example 6 - Introduction of the chimaeric DNA sequence of Example 5 into tobacco

A recombinant Agrobacterium strain was constructed by mobilizing pTTM3 (from Example 5) from E. coli into Agrobacterium C58C1 Rif^R containing pGV2260 (De Blaere et al (1985) NAR 13, 4777). Mobilization was carried out using E. coli HB101 containing pRK2013 (Figurski et al (1979) PNAS 76, 1648) as a helper as described in European patent publication 0,116,718. The resulting Agrobacterium strain contained a hybrid Ti-plasmid comprising pGV2260 and pTTM3.

This strain was used to transform tobacco leaf discs (N. tabacum Petite Havane SR1) using standard procedures as described, for example, in European patent application 87/400,544.0. Transformed calli and

shoots were selected using 5 mg/l of the herbicide phosphinothricin in the medium (De Block et al (1987) EMBO J. 6, 2513). No beta-glucuronidase enzyme activity was detected in the transformed herbicide-resistant calli and shoots.

Then, the transformed shoots were rooted, transferred to soil in the greenhouse and grown until they flowered. The flowers were examined, and only the tapetum cells in the anthers of the stamen were found to contain beta-glucuronidase activity. This shows that the TA29 promoter is capable of directing expression of a heterologous gene, like the beta-glucuronidase gene, selectively in tapetum cells of the plants.

Example 7 - Construction of a chimaeric DNA sequence of PTA29 and a gene 4

A plasmid named "pTTM4", shown in Fig. 6, was constructed by assembling the following well known DNA fragments:

1. a vector fragment, including T-DNA border sequences, derived from pGSC1700 (Cornellisen and Vandewiele (1989) NAR 17 (1), 19-29);
2. the chimaeric sequence (no. 3) of Example 5, containing the PSSU promoter controlling expression of herbicide-resistance gene sfr and the 3' end of a T-DNA gene 7;
3. the chimaeric sequence (no. 4) of Example 5, containing the PNOS promoter controlling expression of the neo gene and the 3' end of the octopine synthase gene; and
4. a chimaeric sequence containing the PTA29 promoter cassette from Example 3, fused in frame with an Agrobacterium T-DNA gene 4 that encodes isopentenyl transferase (Akiyoshi et al (1984) PNAS 76, 5994; Barry et al (1984) PNAS 81, 4776) containing its own 3' end transcription regulation signals.

pTTM4 is a binary type T-DNA vector containing, within the T-DNA border sequences, the following chimaeric sequences: PSSU-sfr and PNOS-neo in which the sfr and neo genes are marker DNAs that encode dominant selectable markers for plants and that are under control of respectively PSSU and PNOS as second promoters; and PTA29-gene 4 in which gene 4 is a male-sterility DNA that is under the control of PTA29 as a first promoter and encodes the enzyme isopentenyl transferase which will cause the enhanced production of cytokinin. Enhanced cytokinin production in tapetum cells, under the control of the TA29 promoter, will disturb the metabolism and organogenesis of the tapetum cells.

Example 8 - Introduction of the chimaeric DNA sequence of Example 7 into tobacco

As described in Example 6, pTMM4 (from Example 7) was introduced with mobilization from E. coli into Agrobacterium C58C1 Rif^R. The resulting Agrobacterium strain contained a binary type Ti-plasmid comprising pGV2260 and pTTM4.

As also described in Example 6, this strain was used to transform tobacco leaf discs, and transformed calli and shoots were selected using 5 mg/l of phosphinothricin. Transformed herbicide-resistant shoots were rooted, which shows that gene 4 was not yet being expressed in the transformed plants.

The plants were then transferred to soil in the greenhouse and grown until they flower. The flowers are examined, and no functional tapetum cells are found in their anthers of their stamen. This shows that the TA29 promoter is capable of directing expression of the heterologous gene 4 selectively in tapetum cells of the plants.

Example 9 - Construction of a chimaeric DNA sequence of PTA29 and a RNAse T1 gene

A plasmid named "pTTM6", shown in Fig. 7A, was constructed by assembling the following well known DNA fragments:

1. a vector fragment, including T-DNA border sequences, from pGSC1600;
2. the chimaeric sequence (no. 3) of Example 5, containing the PSSU promoter, the herbicide resistance gene sfr and the 3' end of the T-DNA gene 7; and
3. a chimaeric sequence, containing the pTA29 promoter cassette from Example 3, fused in frame with a synthetic gene encoding RNase T1 from A. orhyzae, (Quaas et al, "Biophosphates and their Analogues-Synthese, Structure, Metabolism and Activity" (1987) Elsevier Science Publisher B.V., Amsterdam; Quaas et al (1988) Eur. J. Biochem. 173, 617-622.) and the 3' end signals of a nopaline synthase ("NOS") gene (An et al (1985) EMBO J. 4 (2), 277).

pTTM6 is a T-DNA vector containing, within the T-DNA border sequences, two chimaeric sequences; PSSU-sfr which is a marker DNA under the control of PSSU as a second promoter; and PTA29-RNase T1 gene which is a male-sterility DNA under the control of PTA29 as a first promoter. Expression in tapetum cells of the male-sterility DNA under the control of the TA29 promoter will produce RNase T1 that will be lethal for the cells, since the RNase T1 will degrade the RNA molecules which are indispensable for these cells' metabolism.

Example 10 - Introduction of the chimaeric DNA sequence of Example 9 into tobacco

As described in Example 6, a recombinant Agrobacterium strain was constructed by mobilization of pTTM6 (from Example 9) from E. coli into Agrobacterium C58C1 Rif^R. The resulting Agrobacterium strain, harboring a cointegrated Ti-plasmid comprised of pGV2260 and pTTM6, was used for transforming tobacco leaf discs. Transformed calli and shoots were selected using 5 mg/l phosphinothricin. That the RNase T1 gene was not expressed in the transformed herbicide-resistant calli and shoots was shown by their growth.

The transformed shoots were rooted, transferred to soil in the greenhouse and grown until they flowered. The transformed tobacco plants developed normal flowers except for their anthers. The anthers, although of

normal shape, dehisched later in time, compared to the anthers of non-transformed tobacco plants (see Fig. 11). Upon dehiscense, either little or no pollen was released from the transformed plants, and the pollen grains formed by the transformed plants, were about 50 to 100 times smaller in volume than normal pollen grains and were irregularly shaped. Moreover, most of the pollen grains from transformed plants failed to germinate, and the germination efficiency of pollen from transformed plants was about 0 to 2% of the germination efficiency of normal pollen grains. Furthermore, the transformed plants did not produce any seeds by self-pollination -- neither by natural self-pollination nor by hand-provoked self-pollination.

Microscopic evaluation, by thin layer cross section, of a transformed plant showed that no normal tapetum layer was formed and that the pollen sack remained empty (see Fig. 12). This shows that the TA29 promoter is capable of directing expression of the heterologous RNase T1 gene selectively in tapetum cells of the transformed plants, and that the RNase T1 is capable of sufficiently disturbing the functioning of the tapetum cells, so as to render the plants male-sterile.

Example 11 - Introduction of a derivative of the chimaeric DNA sequence of Example 9 into oilseed rape

A recombinant Agrobacterium strain was constructed by mobilization of pTTM6A$^-$ from E. coli into Agrobacterium C58 Rif^R containing pMP90 (Koncz and Schell (1986) Mol. Gen. Genetics 204, 383-396). pMP90 provides vir and trans functions and does not carry a gene encoding ampicillin resistance. As shown in Fig. 7B, pTTMA6A$^-$ is a derivative of PTTM6 (from Example 9), in which the β-lactamase gene encoding ampicillin resistance has been inactivated by insertion of a DNA sequence into the ScaI site of the β-lactamase gene.

The resulting Agrobacterium strain (named "A3144"), harboring pMP90 and pTTM6A$^-$, was used for the transformation of Brassica napus according to the procedure of Lloyd et al (1986) Science 234, 464-466 and Klimaszewska et al (1985) Plant Cell Tissue Organ Culture 4, 183-197. Carbenicillin was used to kill A3144 after co-cultivation occurred. Transformed calli were selected on 5 mg/l phosphinotricine and 100 ug/ml kanamycin, and resistant calli were regenerated into plants. After induction of shoots and roots, the transformants were transferred to the greenhouse and grown until they flower. The flowers are examined, and they exhibit essentially the same phenotype as was observed for the transformed tobacco plants described in Example 10. This shows that the TA29 promoter is capable of directing the expression of the heterologous RNase T1 gene selectively in tapetum cells of plants other than tobacco, so as to render such other plants male-sterile.

Example 12 - Construction of a chimaeric DNA sequence of PTA29 and a Barnase gene

A plasmid named "pTTM8" shown in Fig. 8, was constructed by assembling the following well known fragments:

1. a vector fragment, including T-DNA border sequences derived from pGSC1700 (Cornelissen and Vandewiele (1989) NAR 17 (1) 19-29) and in which the β-lactamase gene (1′ of Fig. 8) has been inactivated by insertion of a DNA sequence into its ScaI site;
2. the chimaeric sequence (no. 3) of Example 5, containing the PSSU promoter, the herbicide-resistance gene sfr and the 3′ end of T-DNA gene 7;
3. the chimaeric sequence (no. 4) of Example 5, containing the PNOS promoter, the neo gene, and the 3′ end of the octopine synthase gene; and
4. a chimaeric sequence, containing the PTA29 promoter cassette from Example 3, fused in frame with the Barnase gene from Bacillus amiloliquefaciens (Hartley and Rogerson (1972) Preparative Biochemistry 2, (3), 243-250) and the 3′ end of the nopaline synthase gene of Example 9.

pTTM8 is a binary type T-DNA vector containing, within the T-DNA border sequences, three chimaeric sequences: PSSU-sfr and PNOS-neo which are markers DNAs with respectively PSSU and PNOS as second promoters; and PTA29-Barnase gene which is a male-sterility DNA under the control of PTA29 as a first promoter. Expression in tapetum cells of the male-sterility DNA under the control of the TA29 promoter will produce Barnase selectively in the tapetum cells so that Barnase will interfere with the metabolism of these cells.

Example 13 - Introduction of the chimaeric DNA sequence of Example 12 into tobacco and oilseed rape

As described in Example 11, a recombinant Agrobacterium strain was constructed by mobilizing pTTM8 (from Example 12) from E. coli into Agrobacterium C58C1 Rif^R containing pMP90 (Koncz and Schell (1986) Mol. Gen. Genetics 204, 383-396). The resulting strain (named "A3135"), harboring pMP90 and pTTM8, is used for tobacco leaf disc transformation and for oilseed rape transformation. Transformed calli and shots are selected using 5mg/l phosphinothricin and 100 ug/ml kanamycin. That the Barnase gene is not expressed in the transformed herbicide-resistant calli and shoots is shown by their growth.

The transformed shoots are rooted, transferred to soil in the greenhouse and grown until they flower. The flowers of both the tobacco and oilseed rape are examined, and a phenotype is observed for the transformed plants that is essentially the same as the phenotype of the transformed tobacco plants described in Example 10. This shows that the TA29 promoter is capable of directing expression of the heterologous Barnase gene selectively in tapetum cells of the plants, thereby rendering the plants male-sterile.

Example 14 - Construction of a chimaeric DNA sequence of pTA29 and a gene encoding papain

A plasmid named "pTVEP1", shown in Fig. 9A, is constructed by assembling the following well known fragments:

1. a vector fragment, including T-DNA border sequences derived from pGSC1700 and in which the β-lactamase gene (1′ of Fig. 9A) has been inactivated by insertion of a DNA sequence into its ScaI site;
2. the chimaeric sequence (no. 3) of Example 5, containing the PSSU promoter, the herbicide resistance gene sfr and the 3′ end of T-DNA gene 7.
3. the chimaeric sequence (no. 4) of Example 5, containing the PNOS promoter, the neo gene and the 3′ end of the octopine synthase gene; and
4. a chimaeric sequence, containing the PTA29 promoter cassette from Example 3, fused in frame with:
a) a papain gene from Carica papaya fruit, encoding the papain zymogen which is a plant endopeptidase (Cohen et al (1986) Gene 48, 219-227) capable of attacking peptide, as well as ester, bonds; the following modifications are made in the DNA sequence of Cohen et al (1986) using site directed mutagenesis as described in Example 3:
i. the nucleotide A, position-1 upstream of the first ATG codon, is mutated into nucleotide C in order to obtain a suitable NcoI cloning site; and
ii. the GAA codons encoding glutamate at positions 47, 118, 135, respectively, are mutated into CAA codons encoding glutamine; and
b) the 3′ end of the nopaline synthase gene of Example 9.

pTVEP1 is a binary type T-DNA vector containing, within the T-DNA border sequences, three chimaeric sequences: PSSU-sfr and PNOS-neo which are marker DNAs encoding dominant selectable markers for plant transformations, under the control of respectively PSSU and PNOS as second promoters; and PTA29-Papain gene which is a male-sterility DNA under the control of PTA29 as a first promoter. Expression in tapetum cells of the male-sterility DNA under the control of the TA29 promoter will produce an endopeptidase (the papain zymogen) that will cleave proteins in the tapetum cells, thus leading to the death of these cells.

A plasmid name "pTVEP2", shown in Fig. 9B, is also constructed by assembling the following well known fragments:

1. a vector fragment, including T-DNA border sequences derived from pGSC1700 and in which the β-lactamase gene (1′ of Fig. 9B) has been inactivated by insertion of a DNA sequence into the ScaI site;
2. the chimaeric sequence (no. 3) of Example 5, containing the PSSU promoter, the herbicide resistance gene sfr and the 3′ end of T-DNA gene 7;
3. the chimaeric sequence (no. 4) of Example 5, containing the PNOS promoter, the neo gene, and the 3′ end of the octopine synthase gene; and
4. a chimaeric sequence, containing the PTA29 promoter cassette of Example 3, fused in frame with: a) a papain gene from Carica papaya fruit, encoding the active protein of the papain zymogen; the following modifications are made in the DNA sequence of Cohen et al (1986), using site directed mutagenesis as described in Example 3:
i. the AAT codon encoding Asn, upstream of the first Ile residue of the active protein, is mutated into a GAT codon, which provides a suitable EcoRV cloning site (GAT ATC). The EcoRV engineered site is fused directly to the pTA29 cassette in order to obtain a direct in frame fusion of the promoter with the sequence encoding the active protein of the papain zymogen; and
ii. the GAA codons encoding glutamate at positions 47, 118, 135 respectively, are mutated into CAA codons encoding glutamine; and
b) the 3′ end of the nopaline synthase gene of Example 9.

pTVEP2, like pTVEP1, is a binary type T-DNA vector containing, within the T-DNA border sequences, three chimaeric genes: PSSU-sfr and PNOS-neo encoding dominant selectable markers for plant transformations; and PTA29-Papain gene which encodes an endopeptidase that will cleave proteins in the tapetum cells, thus leading to the death of these cells.

Example 15 - Introduction of the chimaeric DNA sequences of Example 14 into tobacco and oilseed rape

As described in Example 11, pTVEP1 and pTVEP2, are each mobilized from E. coli into separate Agrobacterium C58C1 Rif^R carrying pMP90.

The resulting strains, harboring pMP90 with pTVEP1 and pMP90 with pTVEP2, are used to transform tobacco and oilseed rape following the procedures of Examples 11 and 13. That the papain genes are not expressed in transformed herbicide- and kanamycin-resistant calli, shoots and roots is shown by their growth.

The transformed plants are transferred into the greenhouse and grown in soil until they flower. The flowers of both the tobacco and oilseed rape are examined, and phenotypes are observed for the transformed plants that are essentially the same as the phenotype of the transformed tobacco plants described in Example 10. This shows that the TA29 promoter is capable of directing expression of the heterologous papain genes in pTVEP1 and pTVEP2 selectively in tapetum cells of the plants, thereby rendering the plants male-sterile.

Example 16 - Construction of a chimaeric DNA sequence of pTA29 and a gene encoding EcoRI

A plasmid named "pTVE63", shown in Fig.10A, was constructed by assembling the following well known fragments:

1. a vector fragment, including T-DNA border sequences derived from pGSC1701A2 (European patent application 87/115985.1);
2. the chimaeric sequence (no. 3) of Example 5, containing the PSSU promoter, the herbicide-resistance gene sfr and the 3′ end of T-DNA gene 7;

3. the chimaeric sequence (no. 4) of Example 5, containing the PNOS promoter, the neo gene and the 3′ end of the octopine synthase gene;

4. a chimaeric sequence, containing the pTA29 promoter cassette of Example 3, fused in frame with:

a) a gene encoding the EcoRI restriction endonuclease from an E. coli (Green et al (1981) J. Biol. Chem. 256, 2143-2153; Botterman and Zabeau (1985) Gene 37, 229-239) and capable of recognizing and cleaving the target sequence GAATTC on a double stranded DNA; the following modifications were made in the DNA sequence of Green et al (1981) using site directed mutagenesis as described in Example 3:

I. the nucleotides of the ATG initiation codon were replaced by ATGCA, creating a NsiI site at the initiation codon and yielding the following nucleotide sequences:

ATGCA,TCT,AAT...; and

II. the HindII-HindIII fragment of the EcoRI gene cloned in pEcoR12 (Botterman and Zabeau, 1985) was cloned into the pMAC5-8 site directed mutagenesis vector; and

b) the 3′ end of the nopaline synthase gene of Example 9; and

5. a gene encoding an EcoRI methylase under the control of the its natural promoter (Botterman and Zabeau (1985) Gene 37, 229-239) which is capable of inhibiting the activity of EcoRI in E. coli or Agrobacterium, in order to overcome potential leaky expression of the EcoRI gene in microorganisms.

pTVE63 is a binary type T-DNA vector containing, within the T-DNA border sequences, three chimaeric sequences: PSSU-sfr and PNOS-neo which are marker DNAs under the control of respectively PSSU and PNOS as second promoters; and PTA29-EcoRI gene which is a male-sterility DNA under the control of PTA29 as a first promoter. Expression of the male-sterility DNA under the control of the TA29 promoter in tapetum cells will produce the EcoRI restriction endonuclease which will cleave double stranded DNA at the GAATTC sites (see for review of type II restriction modification systems: Wilson (1988) TIG4 (11), 314-318) of the tapetum cells, thus leading to the death of these cells.

A plasmid named pTVE62, shown in Fig. 10B, was also constructed by assembling the following well known fragments:

1. a vector fragment, including T-DNA border sequences derived from pGSC1701A2;

2. the chimaeric sequence (no. 3) of Example 5, containing the PSSU promoter, the herbicide-resistance gene sfr and the 3′ end of T-DNA gene 7;

3. the chimaeric sequence (no. 4) of Example 5, containing the PNOS promoter, the neo gene and the neo 3′ end of the octopine synthase gene;

4. a chimaeric sequence, containing the pTA29 promoter cassette of Example 3, fused in frame with a gene fragment encoding the transit peptide of the Mn-superoxide dismutase ("Mn-SOD") which is a NcoI-PstI fragment of a HpaI-HindIII fragment from pSOD1 (Bowler et al (1989) Embo J. 8, 31-38); the following modifications were made in the DNA sequence of Bowler et al using site directed mutagenesis as described in Example 3:

I. the AA nucleotides located upstream at position -2 and -1 of the ATG initiation codon were changed to CC nucleotides creating a NcoI site at the initiation codon and yielding the following nucleotide sequences:

```
- CCATGGCACTAC
     NcoI
```

II. the T,TCG,CTC, nucleotides located immediately downstream of the processing site of the transit peptide were changed to C,TGC,AGC, creating a PstI site behind the processing site and yielding the following nucleotide sequences:

```
                  | L   Q   T   F   S   L
CTC,CGC,GGC,      | TTG,CAG,ACC,TTT,TCG,CTC
CTC,CGC,GGC,      | TTG,CAG,ACC,TTC,TGC,AGC...
                  ▼                   PstI
```

in which the arrow indicates the processing site of the transit peptide sequence and the upper line the aminoacid sequence corresponding with the Mn-SOD coding sequence; the NcoI-PstI fragment was also fused in frame with a gene encoding the EcoRI restriction endonuclease from E. coli (Greene et al (1981) J. Biol. Chem. 256, 2143-2153; Botterman and Zabeau (1985) Gene 37, 229-239) and capable of recognition and cleavage of the target sequence GAATTC on a double stranded DNA, as found in pTVE63; and

b) the 3′ end of the nopaline synthase gene of Example 9; and

5. a gene encoding the EcoRI methylase under the control of its natural promoter (Botterman and Zabeau, 1985) which is capable of inhibiting the activity of EcoRI in E. coli or Agrobacterium, in order to

overcome potential leaky expression of the EcoRI gene in microorganisms, this gene being inserted into the vector fragment outside the border sequences.

pTVE62 is a binary type T-DNA vector containing, within the border sequences, three chimeric sequences: PSSU-sfr and PNOS-NPTII which are marker DNAs under the control of respectively PSSU and PNOS as second promoters; and pTA29-transit peptide EcoRI endonuclease gene which is a male-sterility DNA having PTA29 as a first promoter and a transit peptide-encoding sequence between them. Expression of the male-sterility DNA under the control of the TA29 promoter in tapetum cells will produce a restriction endonuclease which will be targeted into the mitochondria of the tapetum cells and cleave the double stranded DNA at the CAATTC sites in such cells. This will lead to the death of these cells.

Example 17 - Introduction of the chimaeric DNA sequences of Example 16 into tobacco and oilseed rape

As described in Examples 11 and 15, pTVE62 and pTVE63, were mobilized from E. coli into Agrobacterium C58C1 Rif^R carrying pMP90. The resulting strains, harboring pTVE62 with pMP90 and pTVE62 (with pMP90, were used to transform tobacco and are used to transform oilseed rape following the procedures described in Examples 11 and 13. That the EcoRI endonuclease genes were not expressed in transformed herbicide- and kanamycin-resistant calli, shoots and roots is shown by their growth.

The transformed plants are transferred into the greenhouse and grown in soil until they flower. The flowers of both the tobacco and oilseed rape are examined, and phenotypes are observed for the transformed plants that are essentially the same as of the transformed tobacco plants described in Example 10. This shows that the TA29 promoter is capable of directing expression of the heterologous EcoRI endonuclease gene selectively in the tapetum cells of the plants transformed with pTVE62 and pTVE63, thereby rendering the plants male-sterile.

Needless to say, this invention is not limited to the transformation of any specific plant(s). The invention relates to any plant, the nuclear genome of which can be transformed with a male-sterility DNA under the control of a first promoter that can direct expression of the male-sterility DNA selectively in the plant's stamen cells, whereby the plant can be both self-pollinated and cross-pollinated. For example, this invention relates to plants such as potato, tomato, oilseed rape, alfalfa, sunflower, cotton, celery, onion, corn, soybean, tobacco, brassica vegetables and sugarbeet.

Also, this invention is not limited to the specific plasmids and vectors described in the foregoing Examples, but rather encompasses any plasmids and vectors containing the male-sterility DNA under the control of the first promoter.

Furthermore, this invention is not limited to the specific promoters described in the foregoing Examples, such as the TA29 promoter, but rather encompass any DNA sequence encoding a promoter capable of directing expression of the male-sterility DNA selectively in stamen cells. In this regard, this invention encompasses the DNA sequence of the TA29 promoter of Fig. 3A, as well as any equivalent DNA sequences, such as that of the TA13 promoter of Fig. 3B and the TA 26 promoter of Fig. 3C, which can be used to control the expression of the male-sterility DNA selectively in tapetum cells of a plant. Indeed, it is believed that the DNA sequences of the TA29, TA26 and TA13 promoters can be modified by: 1) replacing some codons with others that code either for the same amino acids or for other amino acids; and/or 2) deleting or adding some codons; provided that such modifications do not substantially alter the properties of the encoded promoter for controlling tapetum-specific expression of a male-sterility.

In addition, this invention is not limited to the specific male-sterility DNAs described in the foregoing Examples but rather encompasses any DNA sequence encoding a first RNA, protein or polypeptide which disturbs significantly the metabolism functioning and/or development of a stamen cell in which it is produced, under the control of the first promoter.

Also, this invention is not limited to the specific marker DNAs described in the foregoing Examples but rather encompasses any DNA sequence encoding a second RNA, protein or polypeptide which confers on at least a specific plant tissue or specific plant cells, in which such DNA sequence is expressed, a distinctive trait compared to such a specific plant tissue or specific plant cells in which such DNA sequence is not expressed.

Claims

1. A cell of a plant, the nuclear genome of which is transformed with a foreing DNA sequence, preferably a foreing chimaeric DNA sequence, characterized by:

(a) a male-sterility DNA, encoding a first RNA, protein or polypeptide which, when produced or overproduced in a stamen cell of said plant, disturbs significantly the metabolism, functioning and/or development of said stamen cell; and

(b) a first promoter capable of directing expression of said male-sterility DNA selectively in stamen cells of said plant, preferably in anther, pollen and/or filament cells, particularly in tapetum and/or anther epidermal cells; said male-sterility DNA being in the same transcriptional unit as, and under the control of, said first promoter.

2. The cell of claim 1, wherein said foreign DNA sequence also comprises, preferably in the same genetic locus as said male-sterility DNA:

(c) a marker DNA encoding a second RNA, protein or polypeptide which, when present at least in a

specific tissue or in at least specific cells of said plant, renders said plant easily separable from other plants which do not contain said second RNA, protein or polypeptide at least in said specific tissue or specific cells; and

(d) a second promoter capable of directing expression of said marker DNA at least in said specific tissue or specific cells; said marker DNA being in the same transcriptional unit as, and under the control of, said second promoter.

3. The cell of claim 1 or 2, wherein said foreign DNA sequence further comprises:

(e) a first DNA encoding a transit peptide capable of transporting said first protein or polypeptide into a chloroplast or mitochondria of said stamen cell; said first DNA being in the same transcriptional unit as said male-sterility DNA and said first promoter and between said male-sterility DNA and said first promoter; and/or

f) a second DNA encoding a transit peptide capable of transporting said second protein or polypeptide into a chloroplast or mitochondria of at least said specific tissue or specific cells; said second DNA being in the same transcriptional unit as said marker DNA and said second promoter and between said marker DNA and said second promoter.

4. The cell of anyone of claims 1-3, wherein said male-sterility DNA encodes: an RNase, particularly RNase T1 or Barnase; a DNase, especially an endonuclease, particularly EcoRI; a protease, especially a papain, particularly papain Zymogen or papain active protein; a glucanase; a lipase, particularly phospholipase A_2; a lipid peroxidase; a cell wall inhibitor; a bacterial toxin; or a ribozyme, particularly the ribozyme against mRNA encoded by the TA29 gene, the TA26 gene or the TA13 gene; or is an anti-sense DNA, particularly the antisense DNA of the TA29 gene, the TA26 gene or the TA13 gene.

5. The cell of anyone of claims 1-4, wherein said male-sterility DNA encodes an enzyme which catalyzes the synthesis of a phytohormone, particularly an enzyme encoded by gene 1, gene 2 or gene 4 of Agrobacterium T-DNA.

6. The cell of anyone of claims 2-5, wherein said marker DNA is: an herbicide resistance gene, particularly an sfr or sfrv gene; a gene encoding a modified target enzyme for an herbicide having lower affinity for the herbicide, particularly a modified 5-enolpyruvylshikimate-3 phosphate synthase as a target for glyphosate or a modified glutamine synthetase as a target for a glutamine synthetase inhibitor such as phosphinotricine; a gene encoding a protein or a polypeptide conferring a color to at least said specific tissue or specific cells, particulalry the gene A1 or the GUS gene; a gene encoding a protein or a polypeptide conferring a stress tolerance to said plant, particularly the gene encoding Mn-superoxide dismutase; or a gene encoding a protein or a polypeptide conferring a disease or pest resistance, particularly a gene encoding a Bacillus thuringiensis endotoxin that confers insect resistance or a gene encoding a bactericidal peptide that confers a bacterial resistance.

7. The cell of anyone of claims 1-6, wherein said first promoter is PTA29, PTA26, PTA13 or a promoter of a DNA coding for a tapetum-specific mRNA hybridizable to TA29, TA26 or TA13.

8. The cell of anyone of claims 2-7, wherein said second promoter is: a constitutive promoter, particularly a 35S promoter, a 35S'3 promoter, a PNOS promoter or a POCS promoter; a wound-inducible promoter, particularly a TR1' or TR2' promoter; a promoter which directs gene expression selectively in plant tissue having photosynthetic activity, particularly an SSU promoter; or a promoter which directs gene expression selectively in leaf cells, petal cells or seed cells, particularly seed coat cells.

9. A vector suitable for transforming a cell of a plant, particularly a plant capable of being infected with Agrobacterium, comprising said foreign DNA sequence of anyone of claims 1-8, particularly pTTM4, pTTM6, pTTM6A$^-$, pTTM8, pTVEP1, pTVEP2, pTVE62 or pTVE63.

10. A process for producing a male-sterile plant and reproduction material of said plant, having said foreign DNA sequence of anyone of claims 2-8 stably integrated into the nuclear genome of their cells, whereby said male-sterility DNA is capable of being expressed selectively in stamen cells of said plant to produce said first RNA, protein or polypeptide in said stamen cells and whereby said marker DNA can be expressed in at least said specific tissue or specific cells of said plant to render said plant separable from non-transformed plants, characterized by the non-biological steps of: a) transforming a cell of said plant by introducing said foreign DNA sequence into the nuclear genome of said cell; and then b) regenerating said plant and reproduction materials from said cell.

11. A plant cell culture, containing the plant cell of anyone of claims 1-8.

12. A plant, particularly corn, potato, tomato, oilseed rape, alfafa, sunflower, cotton, celery, onion, clover, soybean, tobacco, brassica vegetables aor sugarbeet, containing the plant cell or anyone of claims 1-8.

13. A seed of a plant of claim 12.

14. A process for producing a hybrid seed, characterized by the steps of: a) cross-pollinating i) a male-sterile plant that contains the foreign DNA sequence of anyone of claims 2-8, including both said second promoter and said marker DNA, especially said marker DNA conferring a resistance to an herbicide, particularly an sfr or srfv gene, stably integrated in the nuclear genome of the cells of said male-sterile plant, with ii) a homozygous male-fertile plant without said marker DNA, especially without said marker DNA conferring said herbicide resistance; and then b) separating said male-fertile plant from said male-sterile plant by taking advantage of the absence of expression of said marker DNA at least in said specific tissue or specific cells of said male-fertile plant.

15. The process for producing a hybrid seed of claim 14, wherein said male-sterile plant contains at least

two different marker DNAs stably integrated into the nuclear genome of its cells and said male-fertile plant contains one, but not the other, of said two marker DNAs; and wherein said male-fertile plant is separated from said male-sterile plant by taking advantage of the absence of expression of said other maker DNA at least in said specific tissue or specific cells of said male-fertile plant; said other marker DNA preferably conferring a resistance to an herbicide.

16. The hybrid seed obtained by the process of claim 14 or claim 15.

17. A hybrid plant obtained by growing the hybrid seed of claim 16.

18. The first promoter of claim 7, particularly PTA29, PTA26 or PTA13.

19. A foreign chimaeric DNA sequence of anyone of claims 1-8, wherein said male-sterility DNA is not naturally found under the control of the first promoter and/or is not naturally found in the same genetic locus as said marker DNA.

20. In a process for producing plant and reproduction material, such as seeds, of said plants including a foreign genetic material stably integrated in nuclear genome thereof and capable of being expressed therein as an RNA, protein or polypeptide, comprising the non-biological steps of: a) producing transformed plants cells or plant tissue including said foreign genetic material from starting plant cells or plant tissue not expressing said RNA, protein or polypeptide, b) producing regenerated plants or reproduction material of said plants or both from said transformed plant cells or plant tissue including said foreign genetic material, and c) optionally, biologically replicating said regenerated plants or reproduction material or both; wherein said step of producing said transformed plant cells or plant tissue including said foreign genetic material is characterized by: transforming the nuclear genome of said starting plant cells or plant tissue with a foreign DNA sequence of anyone of claims 1-8, as well as regulatory elements which are capable of enabling the expression of said foreign DNA sequence in said plant cells or plant tissue, to cause the stable integration of said foreign DNA sequence in transformed plant cells or plant tissue, as well as in plants and reproduction material produced therefrom throughout subsequent generations.

FIG. 1

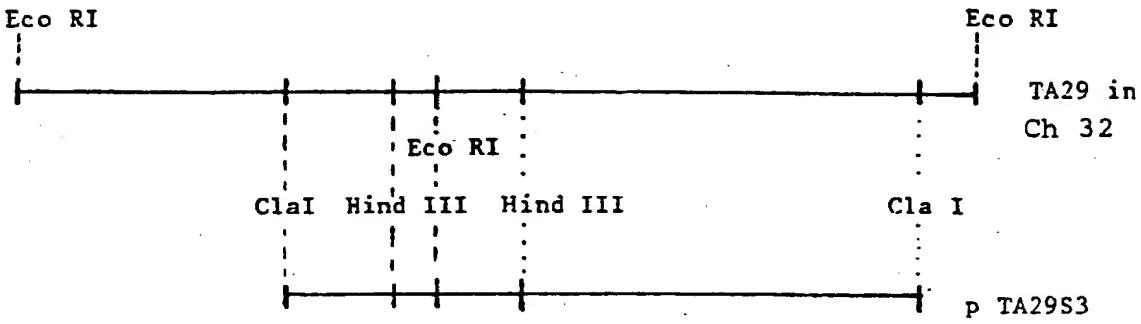

Eco RI
Eco RI
TA29 in
Ch 32
Eco RI
ClaI
Hind III
Hind III
Cla I
p TA29S3

Fig. 2

```
                                   27                                  54
CAA TCC GCT AGA CTA TAC CGT TGC AAG CCA GGG CCA AAT ATG TGT GAC AGT AAA
Q   S   A   R   L   Y   R   C   K   P   G   P   N   M   C   D   S   K

                                   81                                 108
GAC TGT AAT GAG CTT CTC CTA CAC TTT GTT TTC CCA ATG CAA GAC AAA CAT GAC
D   C   N   E   L   L   L   H   F   V   F   P   M   Q   D   K   H   D

                                  135                                 162
AAT AAA CAA GAA CAT CTA AGA TAT GGA GGA CGC CGA GGT ATA GGT CTC ACT GTG
N   K   Q   E   H   L   R   Y   G   G   R   R   G   I   G   L   T   V

                                  189                                 216
GGA GGA GTT GGC GGT TTT GGA ATT GGT TTT GGT GCT TGG GGT GGT GGT GGT GGC
G   G   V   G   G   F   G   I   G   F   G   A   W   G   G   G   G   G

                                  243                                 270
GGA GGA GGT GGT GGT TCT GAT GCC CCT GGT TGT AGT AAC GAT GGC TGT GAC CCT
G   G   G   G   G   S   D   A   P   G   C   S   N   D   G   C   D   P

                                  297                                 324
GGT TTT GGC TGT CCC CCG GGC TGT GGT TAT GCA TGT CCT GCC AAC AAT CCT AGT
G   F   G   C   P   P   G   C   G   Y   A   C   P   A   N   N   P   S

                                  351
GGA GGA ATA ACT GAA TTC CAT ATC TCA GGA TTG TTG GCA
G   G   I   T   E   F   H   I   S   G   L   L   A
```

Figure 3A

ClaI

GTTTGACAGCTTATCATCGATTATATTAGGGATTTTTACACAAATAGCCGGCTATA 56

TTAATTGTTTACTTTTTCTAACCATATACATAGATTATACATTGATTATACATGATTATACACATTTAAT 126

ATATAAATTATGCATATAATATACATTCGCTGGTTATTTTTAGTTTAAGTTATAGGGTGGGAGGCTATTT 196

GGATTAATTCTTTATTATATTAAATATTTACGAATTTCGTGTTTGACGGTGTAAATCATATGTATTGATA 266

CTTGTTGCTTCTTTTATTAATTTTAGTGATTTAGATTCCCTAGAAACTACCATATGCTGTTTTTAGGTTC 336

GTATAATATGAGAAAAGTTTATTTTTAGTCGCTTCCAAATATATATTTATATACTTTCTCCGGTCCACAA 406

TAAGTGATTTTTTGGTTGTTTTCACACAGATTAAGAAATTCACATTTTAACATTAAATAGCAATGAAATT 476

GATCATATTAACCTTTACTATTTCTTCACATAAACATTTCTAACACATACTCCAACACCATTTACTCCAA 546

GGGCACTGTAGTAAAAAAATAATTAAATCATTTTTGAAATCTAAAAAACTCACTTATTTTGGACCATAAA 616

AAAAGGGCCAAAAAATAACTTATTGTGGACCGGAGAGAGTAATACACTTTTTGGTTAGCGAATGCAATTA 686

ATTTAGACATTGTGTTATGTTCCAGTTAACCGCTTCCCTGCACTTCTTTCAATCTATCTCTCGATAGAAA 756

ATTGTGATACTTTGCGACTTCTATCAGAGGACTTTTTGTTTTCCATGTAACAATCTGTCATTTTCGATGG 826

GGAGATTTGCACAAATAGGCTATTTATGTGTCCCAATTTAAATTTTAACCCCATGTCGATCAGAACTTAG 896

CCACGAGCACCAGAAGTTTGATGGATATGTGACTTTGTCACTATCCGGTTTACTAATCAAGAGCTATTTT 966

TATTCAAAATTGGATATCTAGCTAAGTATAACTGGATAATTTGCATTAACAGATTGAATATAGTGCCAAA 1036

CAAGAAGGGACAATTGACTTGTCACTTTATGAAAGATGATTCAAACATGATTTTTTATGTACTAATATAT 1106

ACATCCTACTCGAATTAAAGCGACATAGGCTCGAAGTATGCACATTTAGCAATGTAAATTAAATCAGTTT 1176

TTGAATCAAGCTAAAAGCAGACTTGCATAAGGTGGGTGGCTGGACTAGAATAAACATCTTCTCTAGCACA 1246

GCTTCATAATGTAATTTCCATAACTGAAATCAGGGTGAGACAAAATTTTGGTACTTTTTCCTCACACTAA 1316

GTCCATGTTTGCAACAAATTAATACATGAAACCTTAATGTTACCCTCAGATTAGCCTGCTACTCCCCATT 1386

****

TTCCTCGAAATGCTCCAACAAAAGTTAGTTTTGCAAGTTGTTGTGTATGTCTTGTGCTCTATATATGCCC 1456

* CTTTAATCGATTT

TTGTGGTGCAAGTGTAACAGTACAACATCATCACTCAAATCAAAGTTTTTACTTAAAGAAATTAGCTAAA 1526

TACCATCGAGG HindIII

ATGGTAGCTCCAAAATGGGTTTTCATTTCTTTTATGATTTTGCTAAGCTTAGCAATATGCTCTGGCCAGC 1596

M V A P K W V F I S F M I L L S L A I C S G Q P

CTGTTACCTCTGATGCAATTAAGGCTAAGGAAGCTGATCATGACAACCTCAAAGCTCACACTCTGAGTAA 1666

V T S D A I K A K E A D H D N L K A H T L S N

TATCGACGCCAAAGGCTTTGGAGGAGGCGGTGGATTTGGCATTGGTGGTGGTTGGGCCGGTGGTGGTGGA 1736

I D A K G F G G G G G F G I G G G W A G G G G

GGTGGTGGAGATGGTGGTGGTTCTGACACCCCTAACTACGGTTATAACCCTGGCTGCAGTATCCATGGTT 1806

G G G D G G G S D T P N Y G Y N P G C S I H G C

GCACTGTCCCTGGCTTTGGTTTCCTACCTAAACCTGTCTTTGGTGTCCCAGTCTATTCCCCTGGTTGTGG 1876

T V P G F G F L P K P V F G V P V Y S P G C G

Figure 3 A (continued)

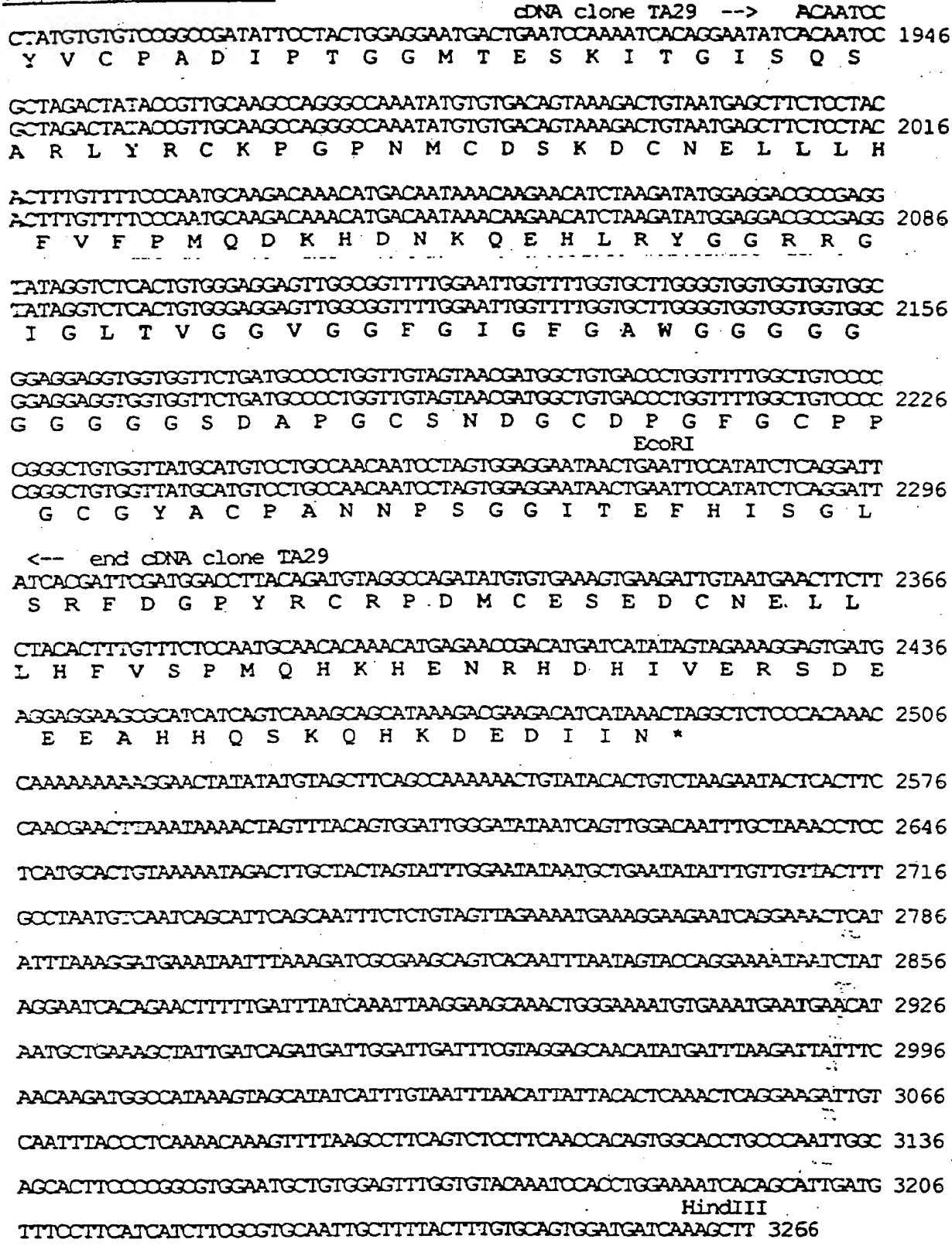
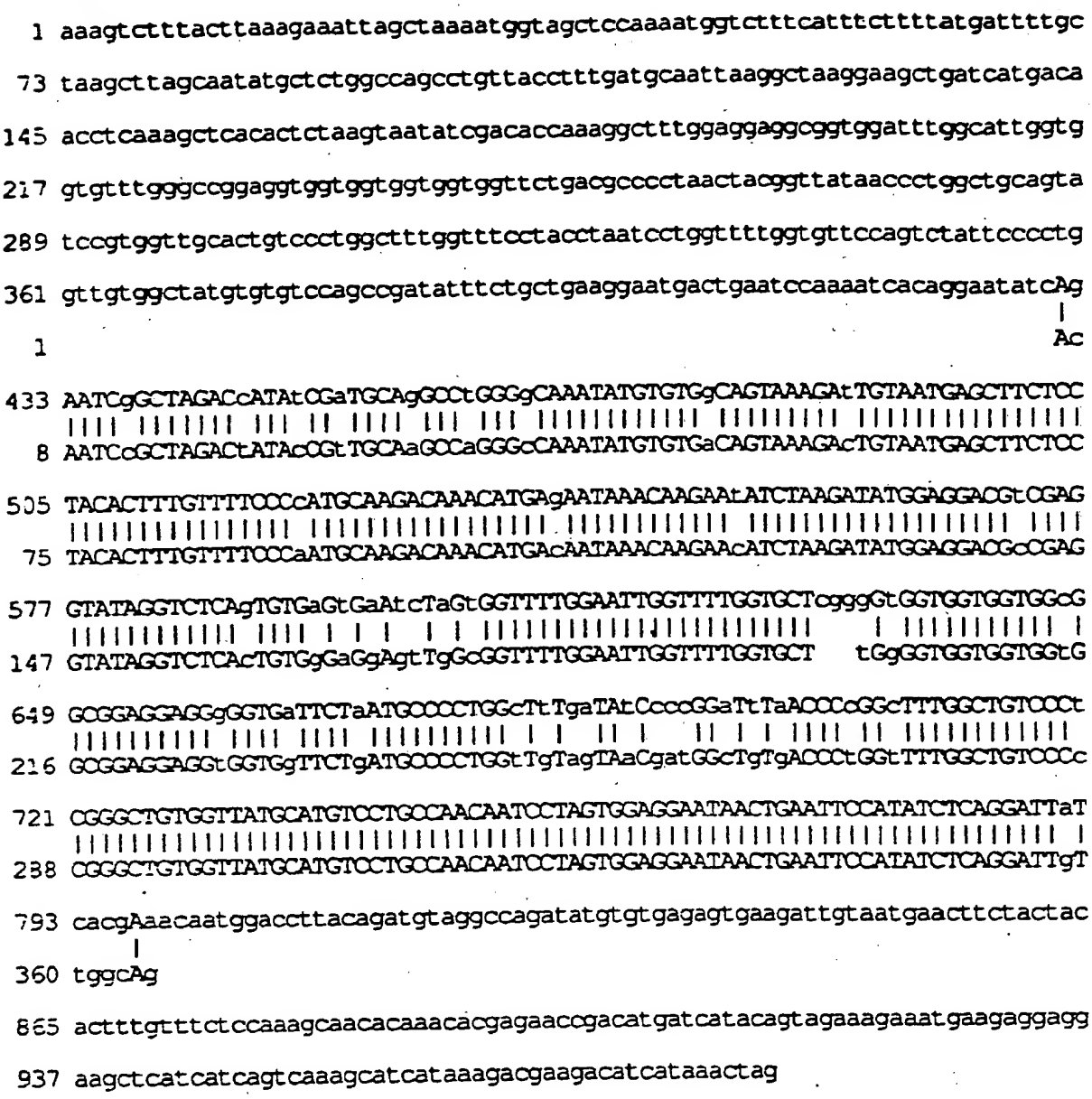


Figure 3 B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145 acctcaaagctcacactctaagtaatatcgacaccaaaggctttggaggaggcggtggatttggcattggtg
217 gtgtttgggccggaggtggtggtggtggttctgacgcccctaactacggttataaccctggctgcagta
289 tccgtggttgcactgtccctggctttggtttcctacctaatcctggttttggtgttccagtctattcccctg
361 gttgtggctatgtgtgtccagccgatatttctgctgaaggaatgactgaatccaaaatcacaggaatatcAg
1 Ac
433 AATCgGCTAGACcATAtCGaTGCAgGCCtGGGgCAAATATGTGTGgCAGTAAAGAtTGTAATGAGCTTCTCC
8 AATCcGCTAGACtATAcCGtTGCAaGCCaGGGcCAAATATGTGTGaCAGTAAAGAcTGTAATGAGCTTCTCC
505 TACACTTTGTTTTCCCcATGCAAGACAAACATGAgAATAAACAAGAAtATCTAAGATATGGAGGACGtCGAG
75 TACACTTTGTTTTCCCaATGCAAGACAAACATGAcAATAAACAAGAAcATCTAAGATATGGAGGACGcCGAG
577 GTATAGGTCTCAgTGTGaGtGaAtcTaGtGGTTTTGGAATTGGTTTTGGTGCTcgggGtGGTGGTGGTGGcG
147 GTATAGGTCTCAcTGTGgGaGgAgtTgGcGGTTTTGGAATTGGTTTTGGTGCT tGgGGTGGTGGTGGtG
649 GCGGAGGAGGgGGTGaTTCTaATGCCCCTGGcTtTgaTAtCcccGGaTtTaACCCcGGcTTTGGCTGTCCCt
216 GCGGAGGAGGtGGTGgTTCTgATGCCCCTGGtTgTagTAaCgatGGcTgTgACCCtGGtTTTGGCTGTCCCc
721 CGGGCTGTGGTTATGCATGTCCTGCCAACAATCCTAGTGGAGGAATAACTGAATTCCATATCTCAGGATTaT
238 CGGGCTGTGGTTATGCATGTCCTGCCAACAATCCTAGTGGAGGAATAACTGAATTCCATATCTCAGGATTgT
793 cacgAaacaatggaccttacagatgtaggccagatatgtgtgagagtgaagattgtaatgaacttctactac
360 tggcAg
865 actttgtttctccaaagcaacacaaacacgagaaccgacatgatcatacagtagaaagaaatgaagaggagg
937 aagctcatcatcagtcaaagcatcataaagacgaagacatcataaactag

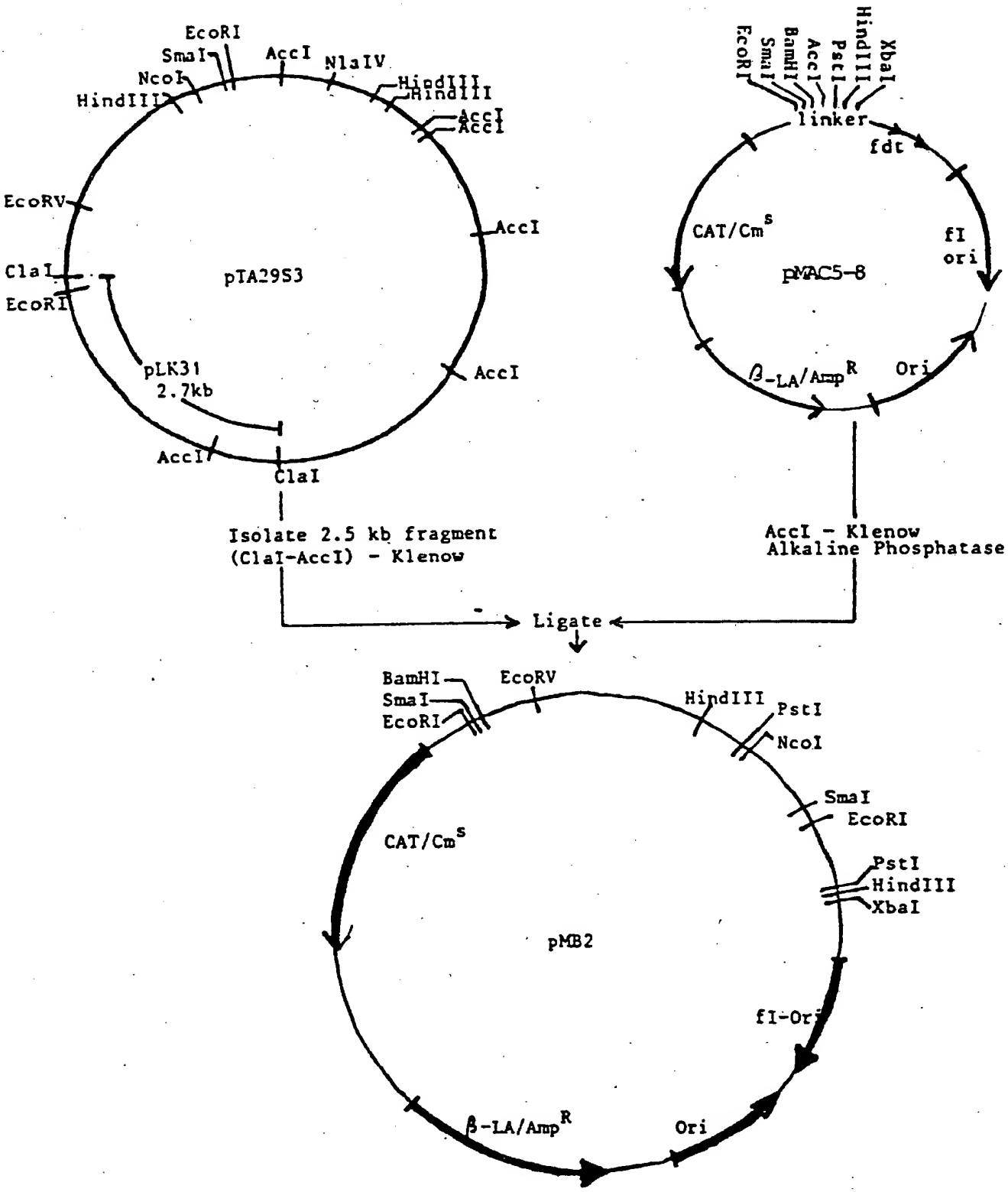
SEQUENCE OF cDNA TA26

```
        10         20         30         40         50         60         70
CGCAGGGGGG GGGGGGGGGG GGGGGGCAAA GATGTCAATA ACTTCCAAGT TCTTGCTAGT TATGTCCCTA

        80         90        100        110        120        130        140
GGACTAATAG TTTTTACCAC ATTTTCACTT GCTGATCAAC ACTACCAATC TACCAAACAT GAGCTTGGAC

       150        160        170        180        190        200        210
GTTCTGATAC TAATCAGCTA AACATGAATG GTTACTTAGC CATGGAACCA GCACCACCAG ACCTTGAGCA

       220        230        240        250        260        270        280
AGAAGGGCAT ATGTGGCGCT TGAACGACGA CTCGATCGCC ATGGAACCAG CACCACCAGA CCTTGAGCAA

       290        300        310        320        330        340        350
GAAGTGCATA TGTGGCGCTT GAACGACGAC TCGATCGCCA TGGAACCAGC ACCAAGGTTT GAGCTAGAAG

       360        370        380        390        400        410        420
GGCAGAAGCA ACATGAGCAT GAGTCACACT TGAGGCTAGT AACTTAGAAA ACATGATTAG CACCAGAATA

       430        440        450        460        470        480        490
GAATTAACTT GGAAGATGGT GGATTATTGT ACTATAGTCC CTTATTCTAA GTTGTGGATC AATAATAAAG

       500        510        520        530        540        550        560
CTCCATTGTC CTAAATTTCC ATCTGAGTTA AATTATCACC TTATAATTAA GTACCCCCCC CCCCCCCCCC
```

C

FIGURE 3C

Fig. 4 A


EcoRI
SmaI
AccI
NlaIV
NcoI
HindIII
HindIII
HindIII
AccI
AccI
EcoRV
AccI
ClaI
EcoRI
pTA29S3
pLK31
2.7kb
AccI
AccI
ClaI
EcoRI
SmaI
BamHI
AccI
PstI
HindIII
XbaI
linker
fdt
CAT/Cm^S
fI
ori
pMAC5-8
β-LA/Amp^R
Ori
Isolate 2.5 kb fragment
(ClaI-AccI) - Klenow
AccI - Klenow
Alkaline Phosphatase
Ligate
BamHI
SmaI
EcoRI
EcoRV
HindIII
PstI
NcoI
SmaI
EcoRI
CAT/Cm^S
PstI
HindIII
XbaI
pMB2
fI-Ori
β-LA/Amp^R
Ori

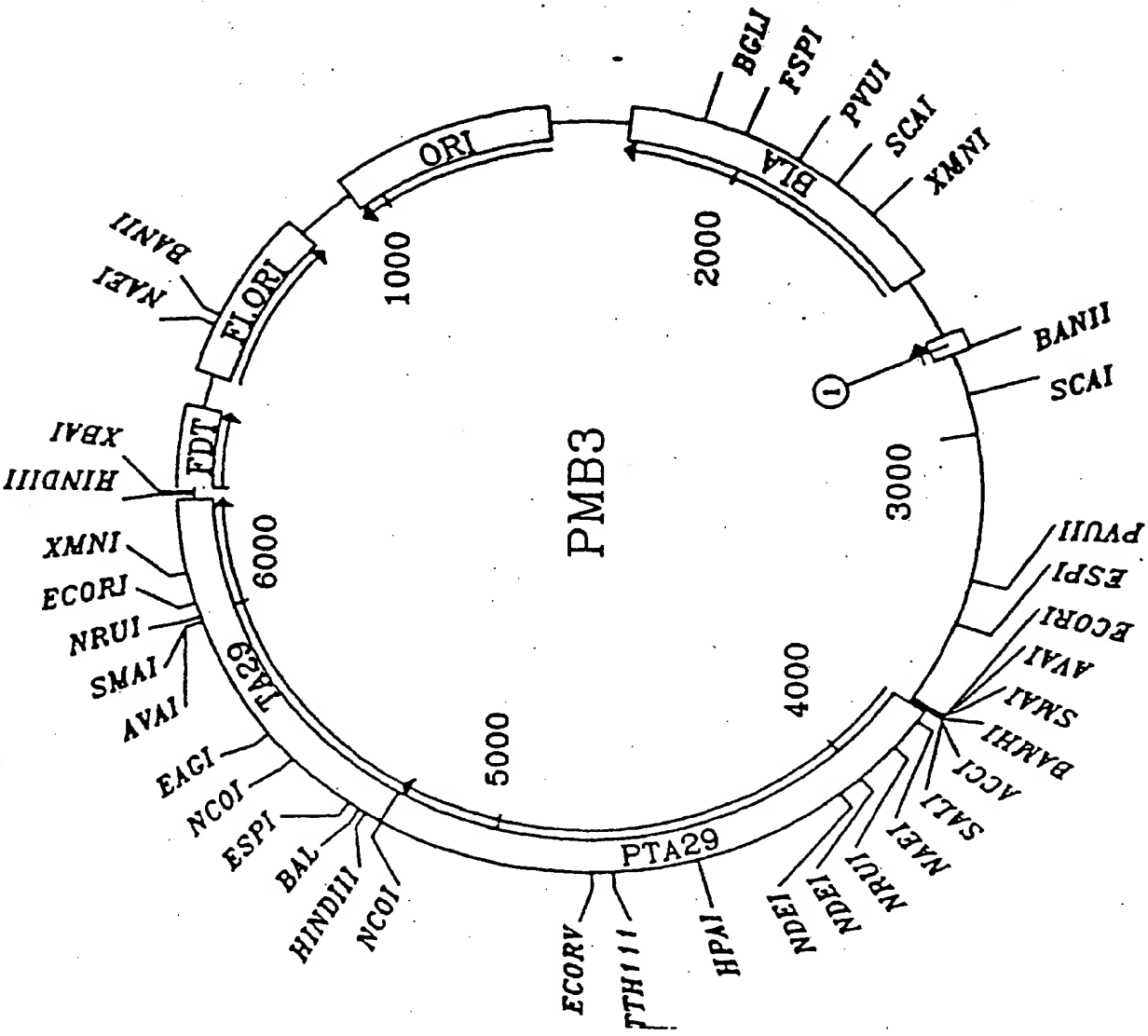


FIG.4 B

LEGEND

① TT

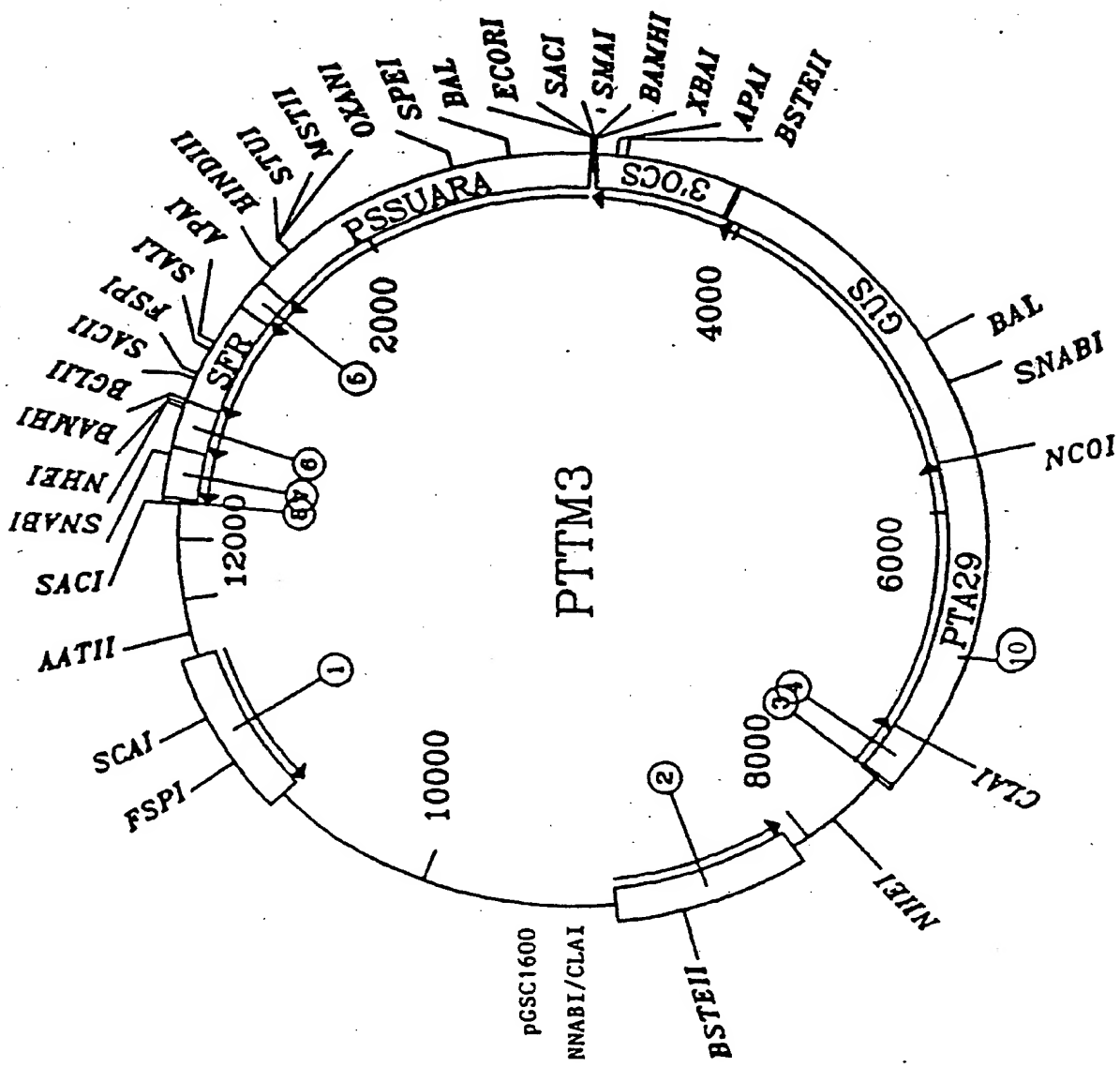


FIG. 5

LEGEND

① BETA-LACTAMASE
② SW-SP-AD.-TRANSF.
③ LB.
④ T-DNA
⑤ TP
⑥ JEND T7
⑦ T-DNA
⑧ RB.
⑩ PTA29S3/pMB3

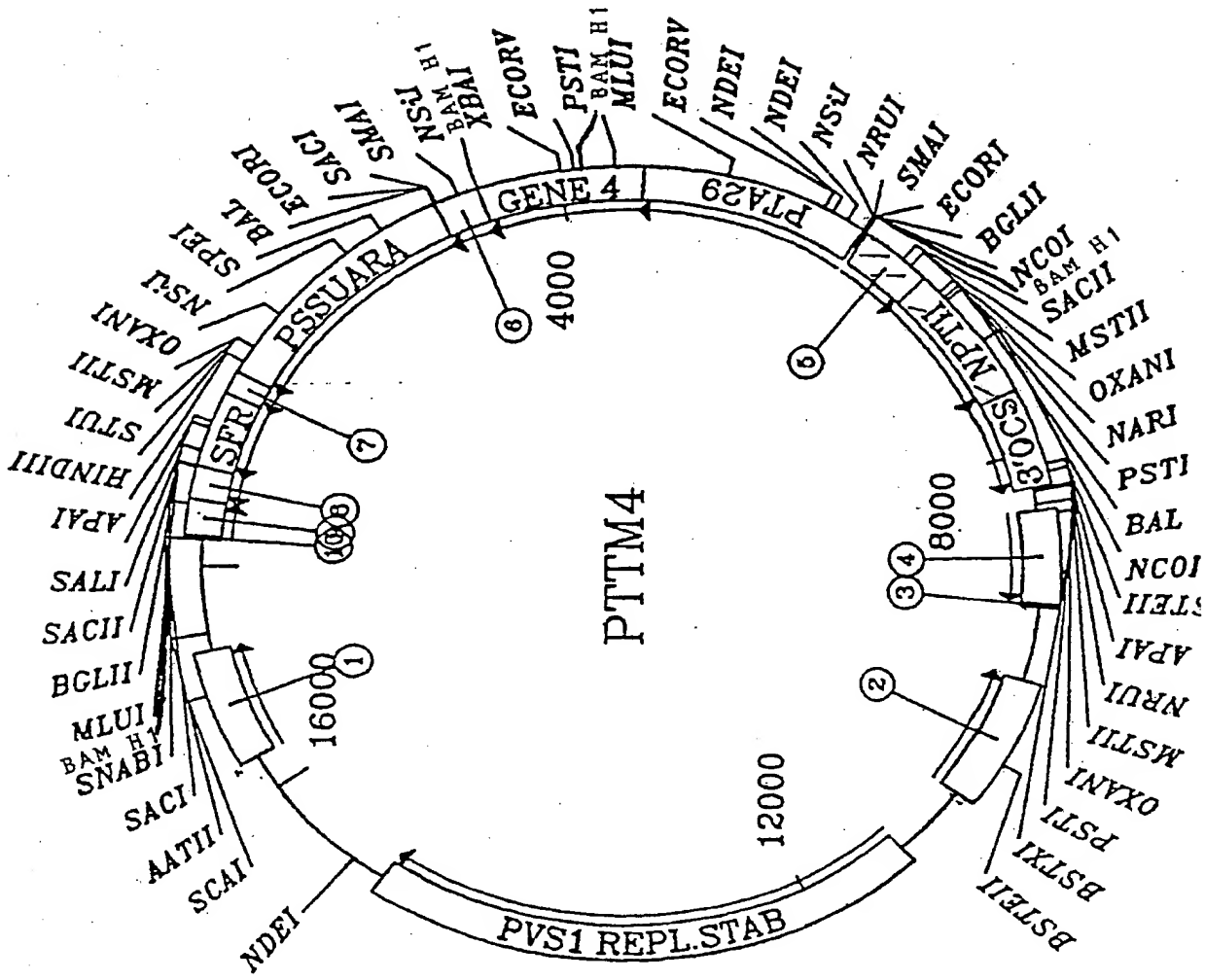


FIG. 6

LEGEND

① BETA-LACTAMASE
② SM-SP-AD TRANSF.
③ LB
④ T-DNA
⑤ PNOS
⑥ 3'NOS
⑦ TP
⑧ 3'END T7
⑨ T-DNA
⑩ RB

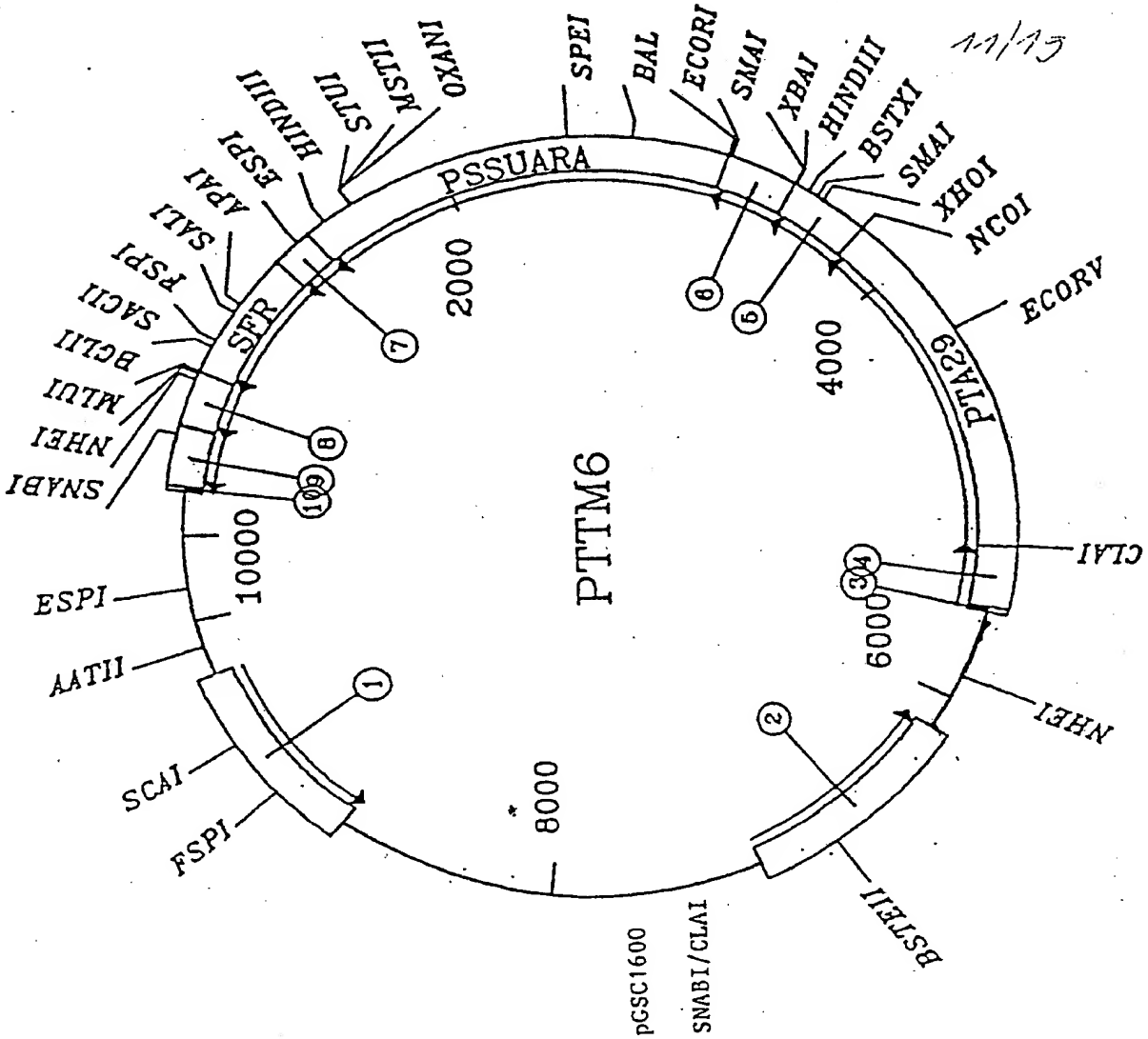


FIG. 7 A

LEGEND

① BETA-LACTAMASE
② SM-SP-AD.TRANSF.
③ LB
④ T-DNA
⑤ RNASE1
⑥ 3'END NOS
⑦ TP
⑧ 3'END T7
⑨ T-DNA
⑩ RB

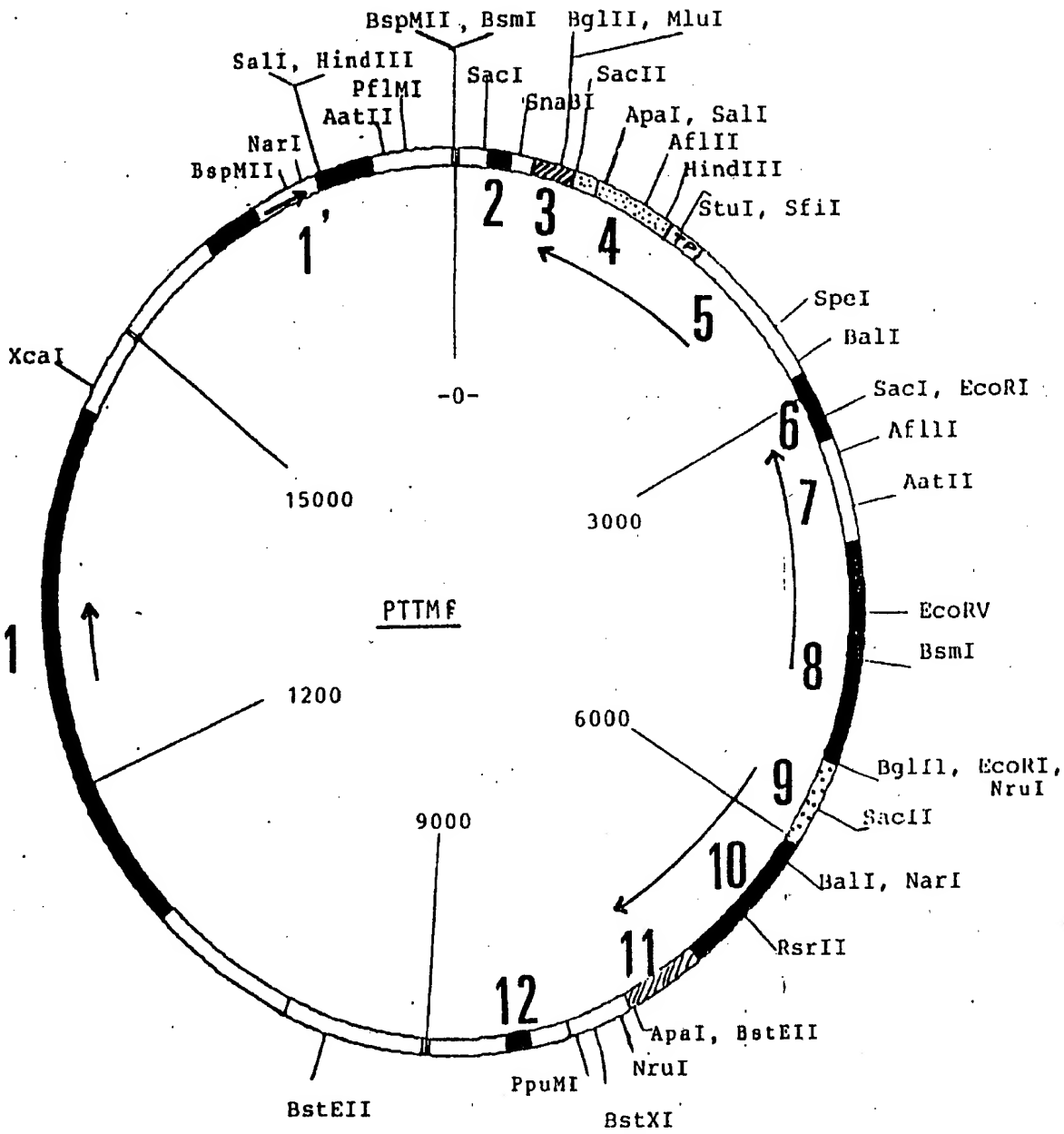


FIGURE 7B
1 : PVS1 ORI
2 : RIGHT BORDER
3 : 3'END T7
4 : SFR
5 : PSSUARA
6 : 3'END NOS
7 : RNASE T1
8 : PTA29
9 : NOS PROMOTOR
10 : NPTII
11 : 3'END OCS
12 : LEFT BORDER
1' : B-LACTAMASE WITH INSERTION

EP 0 344 029 A1

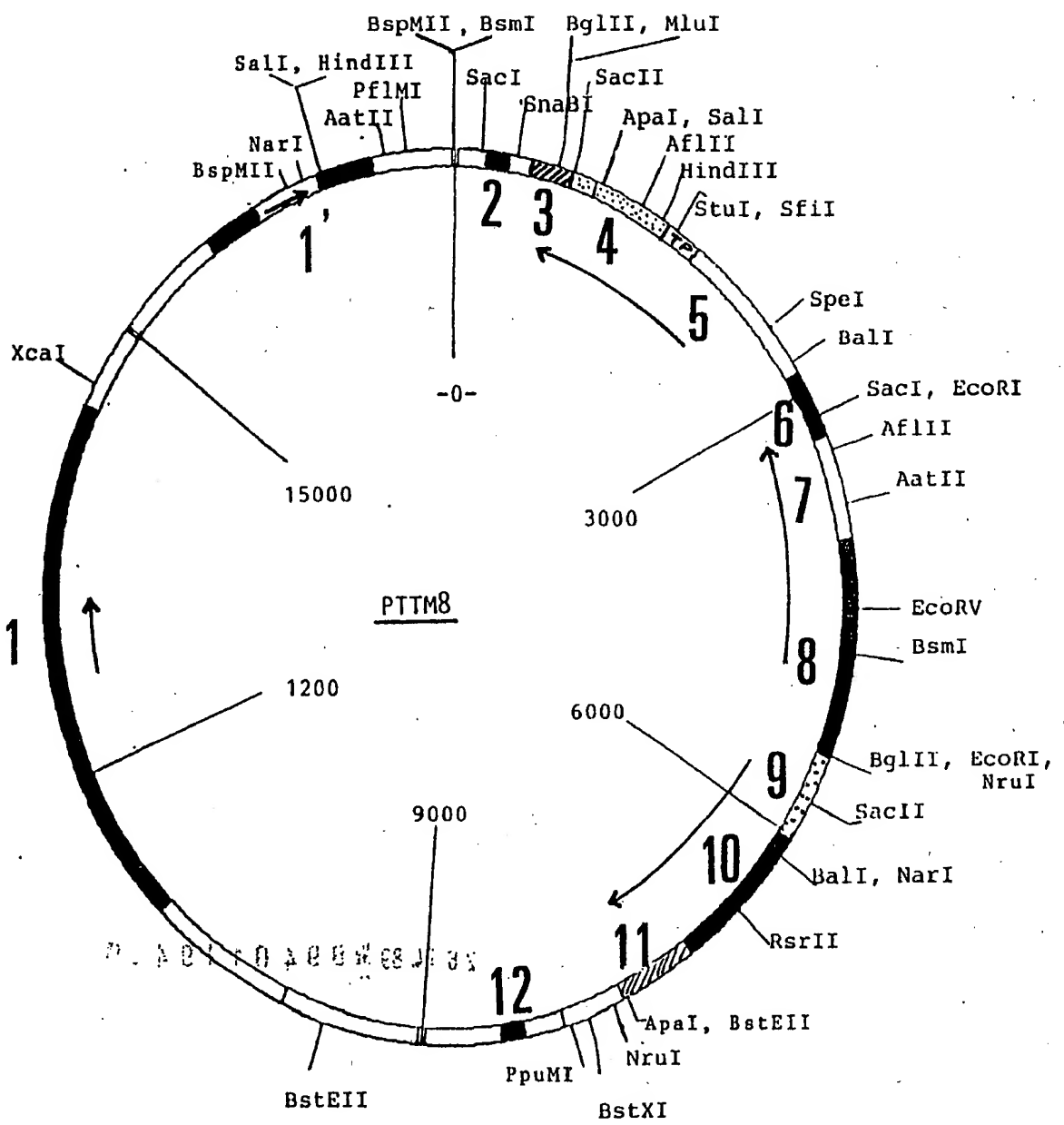


FIGURE 8

1	: PVS1 ORI
2	: RIGHT BORDER
3	: 3'END T7
4	: SFR
5	: PSSUARA
6	: 3'END NOS
7	: BARNASE
8	: PTA29
9	: NOS PROMOTOR
10	: NPTII
11	: 3'END OCS
12	: LEFT BORDER
1'	: B-LACTAMASE WITH INSERTION

EP 0 344 029 A1

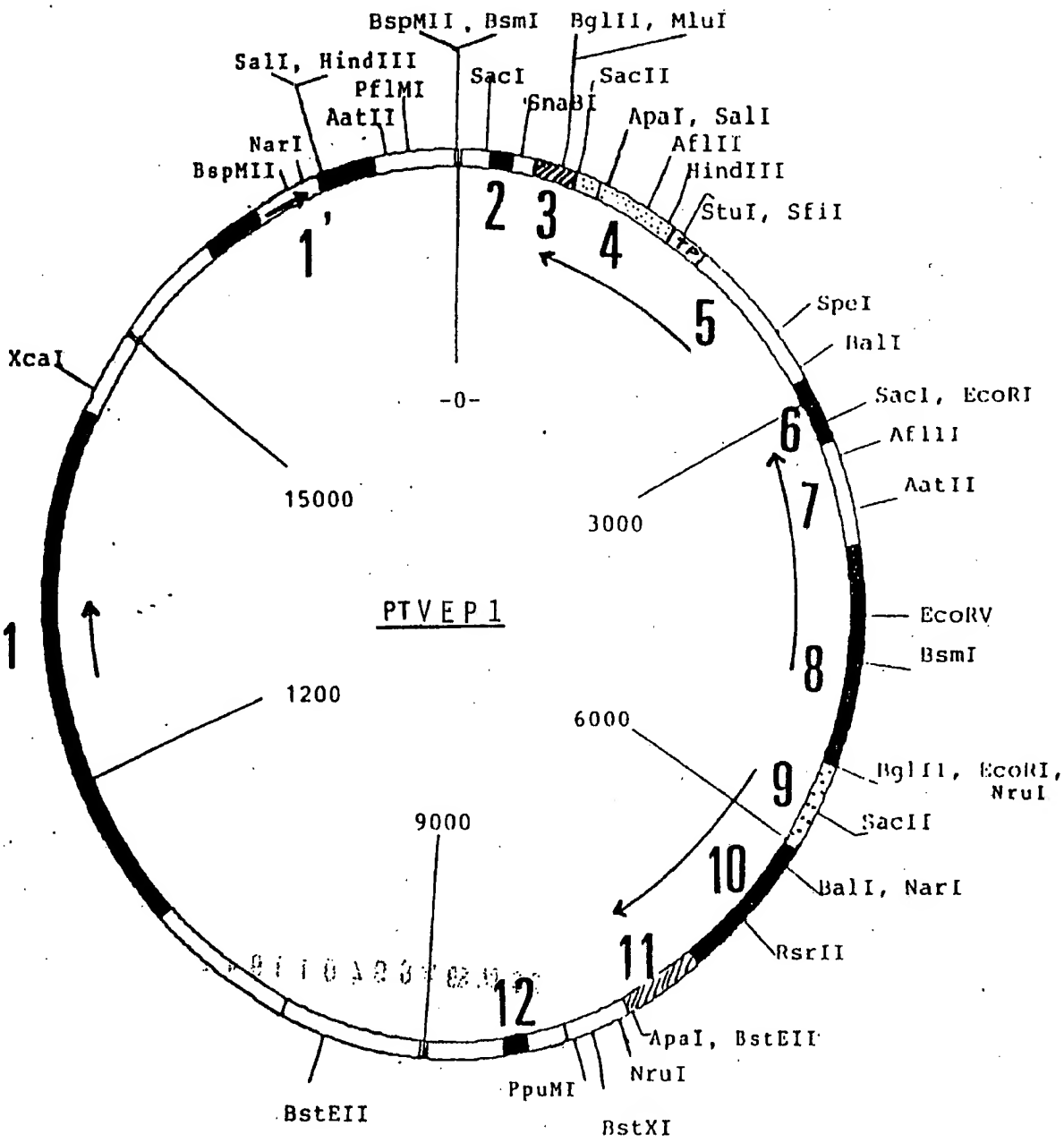


FIGURE 9 A

1 : PVS1 ORI

2 : RIGHT BORDER

3 : 3'END T7

4 : SFR

5 : PSSUARA

6 : 3'END NOS

7 : PAPAIN ZYMOGEN

8 : PTA29

9 : NOS PROMOTOR

10 : NPTII

11 : 3'END OCS

12 : LEFT BORDER

1' : B-LACTAMASE WITH INSERTION

EP 0 344 029 A1

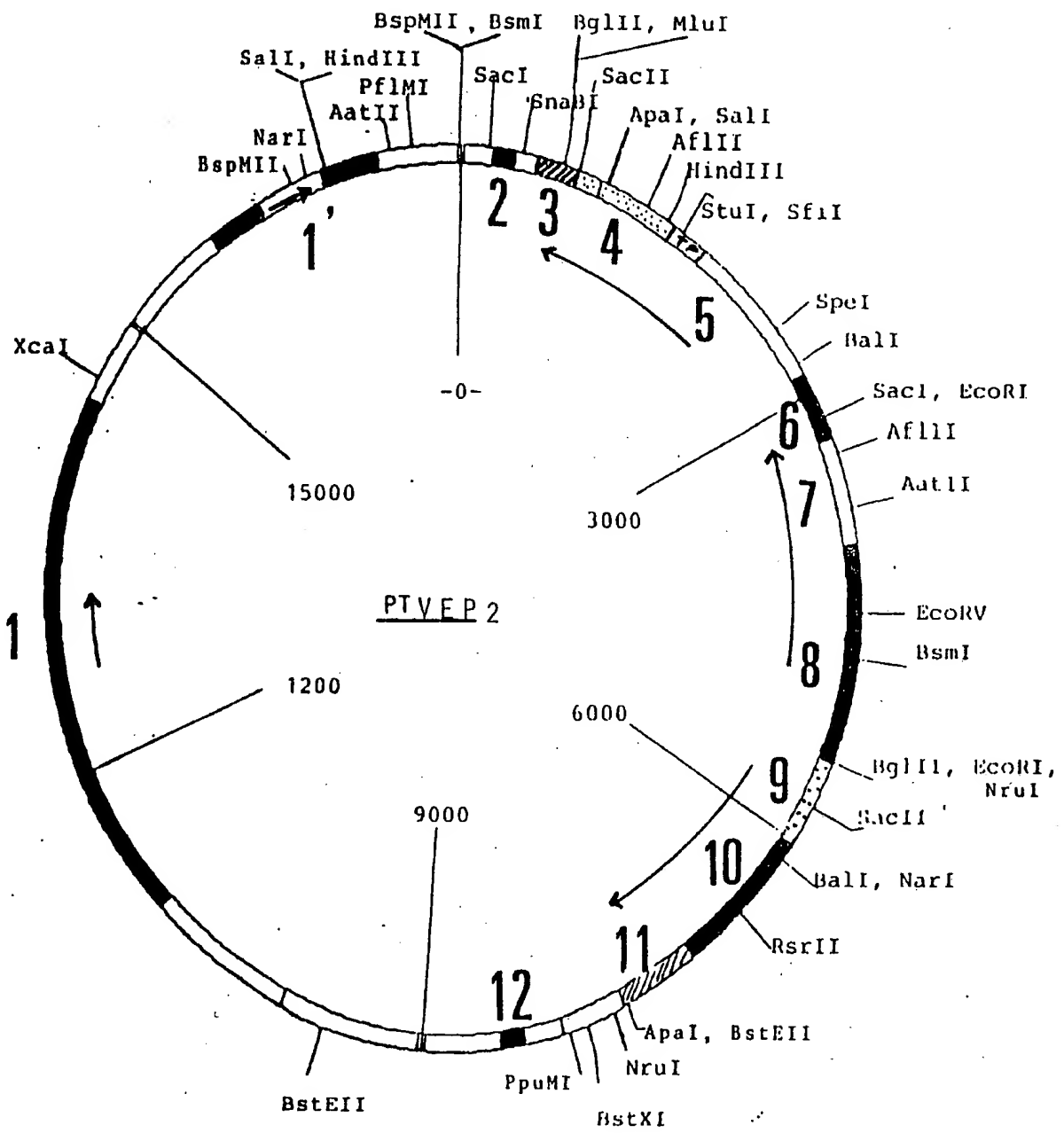


FIGURE 9 B

1	: PVS1 ORI
2	: RIGHT BORDER
3	: 3'END T7
4	: SFR
5	: PSSUARA
6	: 3'END NOS
7	: PAPAIN ACT. PROTEIN
8	: PTA29
9	: NOS PROMOTOR
10	: NPTII
11	: 3'END OCS
12	: LEFT BORDER
1'	: B-LACTAMASE WITH INSERTION

EP 0 344 029 A1

FIGURE 10 A

1 : PVS1 ORI
2 : PBR ORI
3 : RIGHT BORDER
4 : 3' END GENE 7
5 : SFR GENE WITH TRANSIT PEPTIDE
6 : PSSUARA
7 : 3' END NOS
8 : EcoRI
9 : PTA29
10 : NOS PROMOTOR
11 : NPTII
12 : 3' END OCS
13 : LEFT BORDER
14 : METHYLASE RI
15 : SM/SP

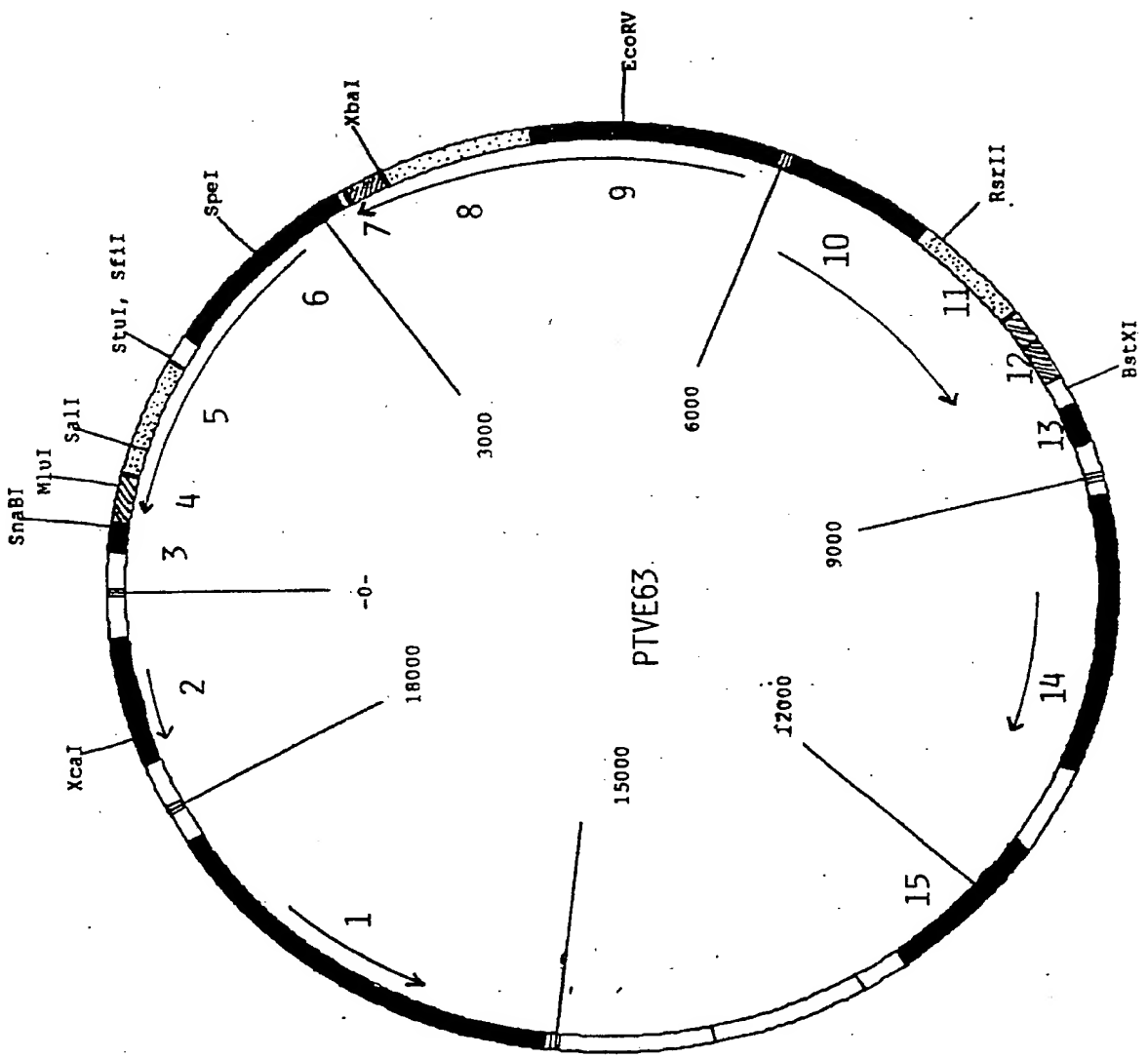
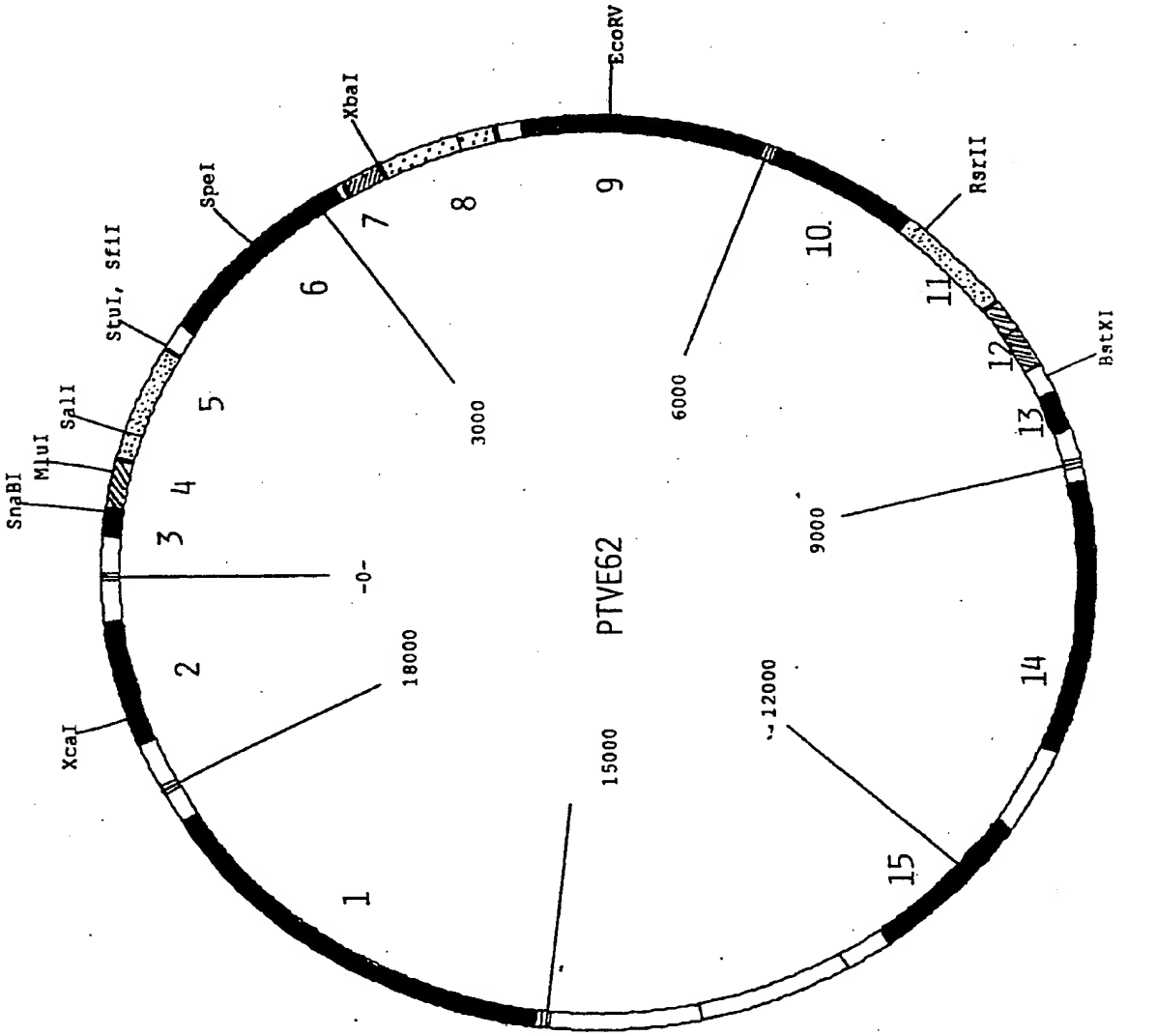


FIGURE 10 B

1 : PVS1 ORI
2 : PBR ORI
3 : RIGHT BORDER
4 : 3' END GENE 7
5 : SFR GENE WITH TRANSIT PEPTIDE
6 : PSSUARA
7 : 3' END NOS
8 : ECORI WITH TRANSIT PEPTIDE
9 : PTA29
10 : NOS PROMOTOR
11 : NPTII
12 : 3' END OCS
13 : LEFT BORDER
14 : METHYLASE RI
15 : SM/SP



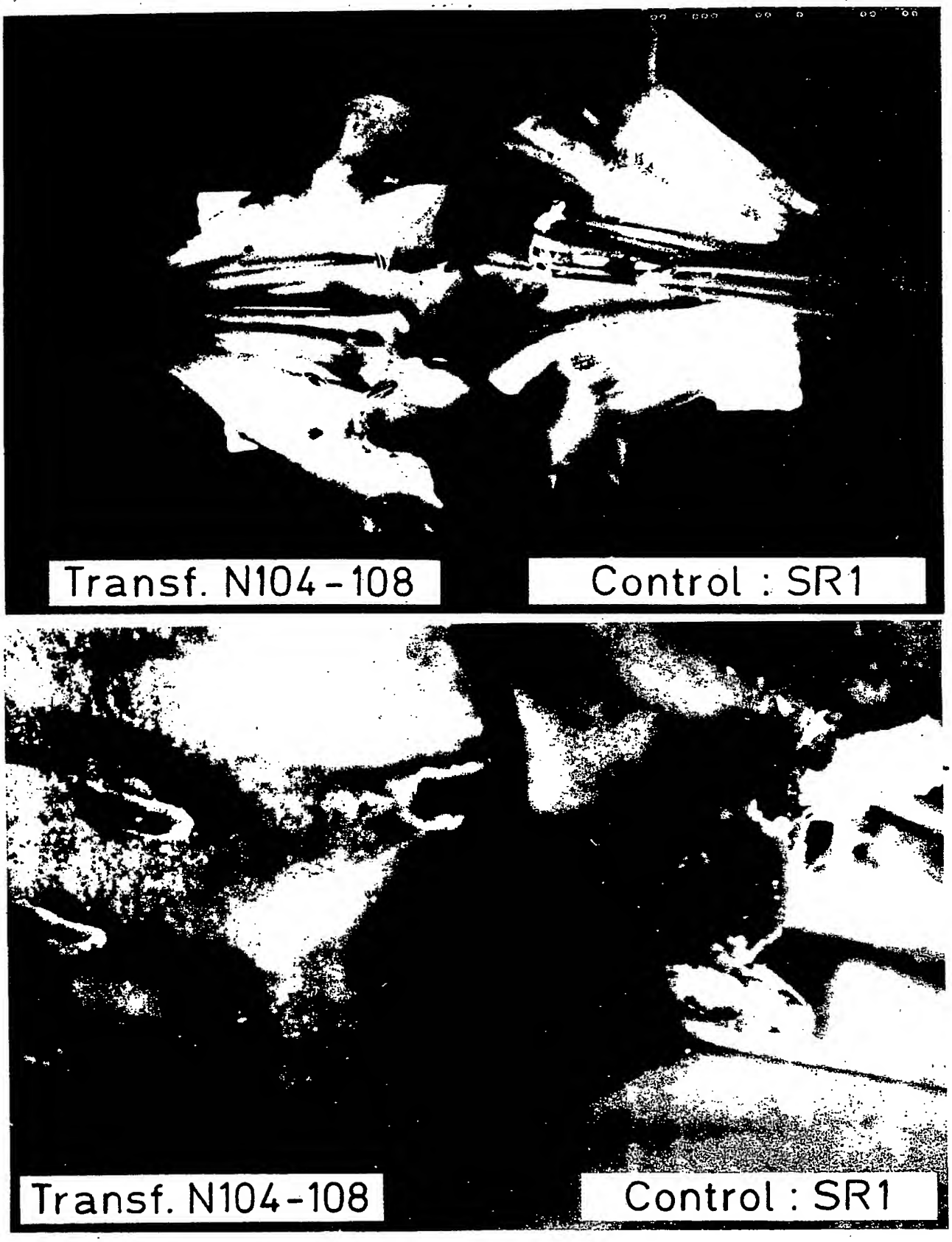
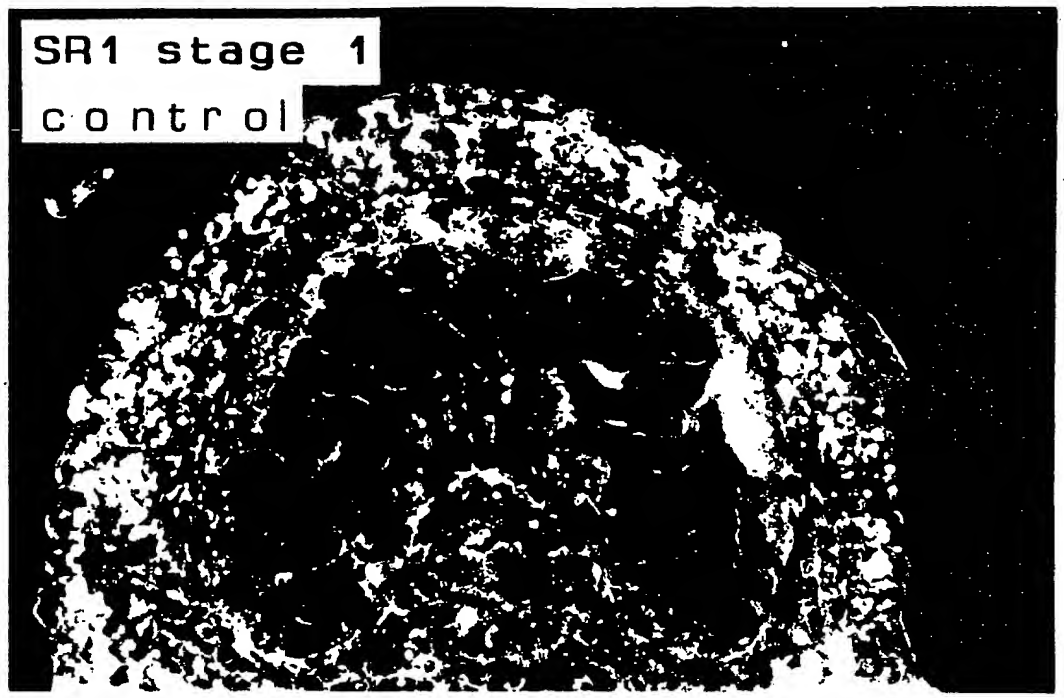
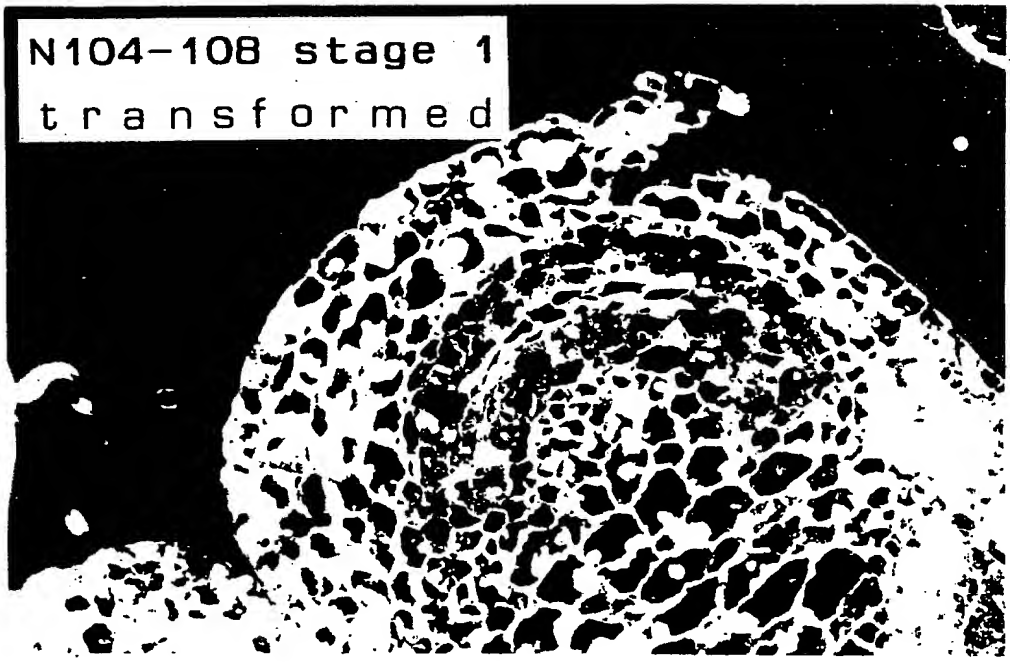


Fig. 11



SR1 STAGE 1, ANTHER CROSS SECTION, THICK TAPETUM AROUND POLLEN SAC, POLLEN STAGE : TETRAD FORM, X250 D.F.



N104-108 STAGE 1, ANTHER CROSS SECTION, THIN LAYER OF TAPETUM, NO POLLEN SAC, NO POLLEN VISIBLE, X250 D.F.

Fig. 12

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 89 40 1194

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
O,P X	JOURNAL OF CELLULAR BIOCHEMISTRY, Supplement 13D, UCLA SYMPOSIA ON MOLECULAR AND CELLULAR BIOLOGY, ABSTRACTS 18TH ANNUAL MEETINGS, 27th March - 7th April 1989, page 312, abstract no. M349, Alan R. Liss, Inc, New York, US; D. TWELL et al.: "Pollen-specific expression directed by chimaeric genes in transgenic tomato and tobacco plants" * Abstract * ---	1,2,5, 10-13, 20	C 12 N 5/00 C 12 N 15/00 A 01 H 1/00
O,P X	JOURNAL OF CELLULAR BIOCHEMISTRY, Supplement 13D, UCLA SYMPOSIA ON MOLECULAR AND CELLULAR BIOLOGY, ABSTRACTS 18TH ANNUAL MEETINGS, 27th March - 7th April 1989, page 292, abstract no. M257, Alan R. Liss, Inc., New York, US; A.J. VAN TUNEN et al.: "Regulation of chalcone flavanone isomerase (CHI) gene-expression in Petunia hybrida: the use of alternative promoters in corolla, anthers and pollen" * Abstract * ---	1,2,4, 10-13, 20	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N A 01 H
O,A	JOURNAL OF CELLULAR BIOCHEMISTRY, Supplement 12C, UCLA SYMPOSIA ON MOLECULAR & CELLULAR BIOLOGY, ABSTRACTS 17TH ANNUAL MEETINGS, 28th February - 10th April 1988, page 137, abstract no. L021, Alan R. Liss, Inc., New York, US; C.S. GASSER et al.: "Analysis of floral specific genes" * Abstract * --- -/-	1-20	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	16-08-1989	MADDOX A.D.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
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O,A	JOURNAL OF CELLULAR BIOCHEMISTRY, Supplement 12C, UCLA SYMPOSIA ON MOLECULAR & CELLULAR BIOLOGY, ABSTRACTS 17TH ANNUAL MEETINGS, 28th February - 10th April 1988, page 168, abstract no. L137, Alan R. Liss, Inc., New York, US; R. WING et al.: "Molecular characterization of pollen specific cDNA clones from tomato (Lycopersicon esculentum)" * Abstract * ---	1-20	
O,A	BIOLOGICAL ABSTRACTS/RRM, abstract no. BR33:81569; S. McCORMICK et al.: "Identification of genes specifically expressed in reproductive organs of tomato", & PLANT BIOLOGY, vol. 4, Tomato Biotechnology; Symposium, Davis, CA, US, August 20-22, 1986 * Abstract * ---	1-20	
A	EP-A-0 198 288 (ADVANCED GENETIC SCIENCES) * Whole document * ---	1-20	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
P,A	SCIENCE, vol. 240, 10th June 1988, pages 1460-1467; R.B. GOLDBERG: "Plants: novel developmental processes" * Page 1464, left-hand column, paragraph 5 - page 1464, right-hand column, paragraph 1 * ---	1-20	
E	EP-A-0 329 308 (PALADIN HYBRIDS) * Whole document *	1,2,4,6,8,10-14,16,17,19,20	
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O,A	JOURNAL OF CELLULAR BIOCHEMISTRY, Supplement 12C, UCLA SYMPOSIA ON MOLECULAR & CELLULAR BIOLOGY, ABSTRACTS 17TH ANNUAL MEETINGS, 28th February - 10th April 1988, page 138, abstract no. L023, Alan R. Liss, Inc., New York, US; J.P. MASCARENHAS: "Characterization of genes that are expressed in pollen" * Abstract * -----	1-20	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

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(54) Title: METHOD TO OBTAIN MALE-STERILE PLANTS

(57) Abstract

A method to obtain male-sterile plants by transforming the nuclear genome of plant cells with a foreign DNA comprising a male-sterility gene comprising: a male-sterility DNA encoding a sterility RNA, protein or polypeptide, preferably barnase, which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell; and, a sterility promoter capable of directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of said plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter; and regenerating plants transformed with said foreign DNA from said transformed cells, which method is characterized by including in said foreign DNA a coregulating gene comprising a coregulating DNA encoding a coregulating RNA, protein or polypeptide, preferably barstar, which is capable, when produced in plant cells wherein said sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of said sterility RNA, protein or polypeptide, said coregulating DNA preferably being under the control of a promoter selected from the group consisting of: a promoter capable of directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells; a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is capable of being placed under control of enhancer elements in the nuclear genome of said plant after integration of said foreign DNA in said plant genome, whereby said coregulating DNA is in a different plant transcriptional unit as said sterility DNA, and provided that, when said coregulating DNA is not under control of a promoter capable of directing expression in plant cells, said coregulating gene is located in said foreign DNA in such a way that after insertion in the plant genome, the coregulating DNA is capable of being placed under the control of plant promoter sequences present in the DNA surrounding said foreign DNA in said plant genome.

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METHOD TO OBTAIN MALE STERILE PLANTS

The present invention relates to an improved method to obtain male-sterile plants using foreign male-sterility genes that comprise plant promoters that direct expression of a male-sterility DNA in stamen cells, and to plants obtained by the method.

Background to the Invention

In many, if not most plant species, the development of hybrid cultivars is highly desired because of their generally increased productivity due to heterosis: the superiority of performance of hybrid individuals compared with their parents (see e.g. Fehr, 1987, Principles of cultivar development, Volume 1 : Theory and Technique, MacMillan Publishing Company, New York; Allard, 1960, Principles of Plant Breeding, John Wiley and Sons, Inc.).

The development of hybrid cultivars of various plant species depends upon the capability to achieve almost complete cross-pollination between parents. This is most simply achieved by rendering one of the parent lines male sterile (i.e. bringing them in a condition so that pollen is absent or nonfunctional) either manually, by removing the anthers, or genetically by using, in the one parent, cytoplasmic or nuclear genes that prevent anther and/or pollen development (for a review of the genetics of male sterility in plants see Kaul, 1988, 'Male Sterility in Higher Plants', Springer Verlag).

For hybrid plants where the seed is the harvested product (e.g. corn, oilseed rape) it is in most cases also necessary to ensure that fertility of the hybrid plants is fully restored. In systems in which the male sterility is under genetic control this requires the existence and use of genes that can restore male fertility. The development of hybrid cultivars is mainly dependent on the availability of suitable and effective sterility and restorer genes.

Endogenous nuclear loci are known for most plant species that may contain genotypes which effect male sterility, and generally, such loci need to be homozygous for particular recessive alleles in order to result in a male-sterile phenotype. The presence of a dominant 'male fertile' allele at such loci results in male fertility.

Recently it has been shown that male sterility can be induced in a plant by providing the genome of the plant with a chimeric male-sterility gene comprising a DNA sequence (or male-sterility DNA) coding, for example, for a cytotoxic product (such as an RNase) and under the control of a promoter which is predominantly active in selected tissue of the male reproductive organs. In this regard stamen-specific promoters, such as the promoter of the TA29 gene of Nicotiana tabacum, have been shown to be particularly useful for this purpose (Mariani et al., 1990, Nature 347:737, European patent publication ("EP") 0,344,029). By providing the nuclear genome of the plant with such a male-sterility gene, an artificial male-sterility locus is created containing the artificial male-sterility genotype that results in a male-sterile plant. Various stamen-specific promoters have been described (see e.g. WO 92/13956, WO 92/13957).

In addition it has been shown that male fertility can be restored to the plant with a chimeric fertility-restorer gene comprising another DNA sequence (or fertility-restorer DNA) that codes, for example, for a protein that inhibits the activity of the cytotoxic product or otherwise prevents the cytotoxic product to be active in the plant cells (EP 0,412,911). For example the barnase gene of Bacillus amyloliquefaciens codes for an RNase, the barnase, which can be inhibited by a protein, the barstar, that is encoded by the barstar gene of B. amyloliquefaciens. The barnase gene can be used for the construction of a sterility gene while the barstar gene can be used for the construction of a fertility-restorer gene. Experiments in different plant species, e.g. oilseed rape, have shown that a chimeric barstar gene can fully restore the male fertility of male sterile lines in which the male sterility was due to the presence of a chimeric barnase gene (EP 0,412,911, Mariani et al., 1991, Proceedings of the CCIRC Rapeseed Congress, July 9-11, 1991, Saskatoon, Saskatchewan, Canada; Mariani et al., 1992, Nature 357:384). By coupling a marker gene, such as a

dominant herbicide resistance gene (for example the bar gene coding for phosphinothricin acetyl transferase (PAT) that converts the herbicidal phosphinothricin to a non-toxic compound [De Block et al., 1987, EMBO J. 6:2513]), to the chimeric male-sterility and/or fertility-restorer gene, breeding systems can be implemented e.g. to select for uniform populations of male sterile plants (EP 0,344,029; EP 0,412,911).

Barnase is an extracellular ribonuclease produced by Bacillus amyloliquefaciens. Barstar is an inhibitor of barnase that is produced intracellularly by the same bacterium to protect it from the toxic effects of the intracellular barnase activity (Hartley, 1989, TIBS, 14:450-454). Initial attempts to clone the barnase gene in E.coli and B.subtilis under control of its own or another bacterial promoter were unsuccesful as the produced barnase proved to be toxic to the host cells. When the barnase gene was reconstructed from previously cloned parts on the same plasmid as the barstar gene, the lethal effects of barnase expression were suppressed (Hartley, 1988, J.Mol.biol. 202:913-915).

Whenever barnase is cloned in a bacterial host cell, such as E.coli, it may be useful to have the barstar gene, under control of its native or another bacterial promoter, present in the host cell to prevent possible harmful effects of undesired barnase expression. Paul et al, 1992, Plant Mol. Biol. 19:611-622 for instance, constructed a chimeric barnase gene under control of a tapetum specific promoter of the A9 gene of Arabidopsis. Plasmids pWP127 and pWP128 contain a DNA fragment encoding barstar and the mature barnase cloned between the 1437 bp A9 promoter fragment and a CaMV polyadenylation sequence. The promoter and coding sequence of barstar were included on these plasmids since mature barnase could not be cloned in its absence in E.coli.

As indicated above barnase DNA has been used to induce male-sterility in plants. However, other uses of barnase have also been described. WO 92/21757 describes inter alia a plant transformed with a nematode-induced chimaeric gene comprising the following operably linked DNA sequences:

- a nematode-induced promoter that is suitable to direct transcription of a foreign DNA substantially selectively in specific root cells, preferably in the cells of fixed-feeding sites of the plant; and,
- a first foreign DNA that encodes barnase;

and which also contains a restorer chimaeric gene, preferably in the same genetic locus as the nematode-induced chimaeric gene, comprising the following operably linked DNA sequences:

- a second promoter, such as a nematode-repressed promoter, which can direct transcription of a second foreign DNA in cells of the plant where the first foreign DNA is expressed, preferably substantially selectively in cells other than the specific root cells, preferably in cells other than the fixed feeding site cells, of the plant, and,
- a second foreign DNA that encodes barstar.

WO 93/19188 describes inter alia a plant transformed with a fungus-responsive chimaeric gene comprising the following operably linked DNA sequences:

- a fungus-responsive promoter that is suitable to direct transcription of a foreign DNA substantially selectively in cells of a plant surrounding, preferably immediately surrounding, a site of infection of the plant by a fungus; and,
- a first foreign DNA that encodes barnase;

and which also contains a restorer chimaeric gene, preferably in the same genetic locus as the fungus-responsive chimaeric gene, comprising the following operably linked DNA sequences:

- a second promoter, such as a constitutive promoter (e.g. 35S), which can direct transcription of a second foreign DNA in cells of the plant other than those surrounding, preferably in at least cells of the plant other than those immediately surrounding, said fungus infection site; and,
- a second foreign DNA that encodes barstar.

A foreign DNA, when introduced in the plant genome appears to integrate randomly in the plant genome. Examination of independently transformed plants has shown a high degree of variability (up to 100-fold) in the expression level of the introduced gene. Several studies have shown no correlation between this "between-transformant variability" and the copy number of the introduced DNA at a given locus.

It has been suggested that some of the variability in expression of introduced genes in transgenic plants is a consequence of "position effects" caused by influences of adjacent plant genomic DNA. Other factors that could contribute to the variability in expression are physiological variability of the plant material, differences in the number of independent T-DNA loci in different transformants or the inhibitory effects of certain T-DNA structures on gene expression. Between-transformant variability in expression has been observed for the majority of introduced genes in transgenic plants. The variability in expression of many introduced genes in independent transgenic plants necessitates large numbers of transgenic plants to be assayed to accurately quantitate the expression of the gene. It would be of great importance if the amount of between-transformant variability could be reduced (Dean et al, 1988, NAR 16:9267-9283).

Summary of the Invention

The invention concerns a plant having in the nuclear genome of its cells foreign DNA comprising :

- a male-sterility gene comprising:
  - a male-sterility DNA encoding a sterility RNA, protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell, and,
  - a sterility promoter directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of the plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter; and
- a coregulating gene comprising:
  - a coregulating DNA encoding a coregulating RNA, protein or polypeptide which is capable, when produced in plant cells wherein the sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of the sterility RNA, protein or polypeptide, and preferably

- a promoter directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells, or
- a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is under control of enhancer elements in the nuclear genome of said plant,

whereby the coregulating DNA is in a transcriptional unit which is different from the transcriptional unit of the sterility DNA.

This invention also provides a method to obtain male-sterile plants which comprises :

- transforming the nuclear genome of plant cells with a foreign DNA comprising a male-sterility gene comprising:
  - a male-sterility DNA encoding a sterility RNA, protein or polypeptide, preferably barnase or a variant thereof, which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell, and,
  - a sterility promoter capable of directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of said plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter, and
- regenerating plants transformed with said foreign DNA from said transformed cells,

which method is characterized by including in said foreign DNA a coregulating gene comprising a coregulating DNA encoding a coregulating RNA, protein or polypeptide, preferably barstar, which is capable, when produced in plant cells wherein said sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of said sterility RNA, protein or polypeptide, said coregulating DNA preferably being under the control of a promoter including :

- a promoter capable of directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells,

- a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is capable of being placed under control of enhancer elements in the nuclear genome of said plant after integration of said foreign DNA in said plant genome,

whereby said coregulating DNA is in a plant transcriptional unit which is different from the plant transcriptional unit of said sterility DNA, and provided that, when said coregulating DNA is not under control of a promoter capable of directing expression in plant cells, said coregulating gene is located in said foreign DNA in such a way that after insertion in the plant genome, the coregulating DNA is capable of being placed under the control of plant promoter sequences present in the DNA surrounding said foreign DNA in said plant genome.

The present invention further provides plants that contain in their nuclear genome said male-sterility gene and said coregulating gene, preferably in the same genetic locus.

Description of the invention

A male-sterile plant is a plant of a given plant species which is male-sterile due to expression of a male-sterility genotype such as a foreign male-sterility genotype containing a male-sterility gene. A restorer plant is a plant of the same plant species that contains within its genome at least one fertility-restorer gene that is able to restore the male fertility to a line of male-sterile plants containing a male-sterility genotype i.e. in those offspring obtained from a cross between a male-sterile plant and a restorer plant and containing both a male-sterility genotype and a fertility-restorer gene. A restored plant is a plant of the same species that is male-fertile and that contains within its genome a male-sterility genotype and a fertility-restorer gene.

A line is the progeny of a given individual plant.

A gene as used herein is generally understood to comprise at least one DNA region coding for an RNA, which may or may not be capable of being translated into a protein or polypeptide, which is operably linked to regulatory sequences that

control the transcription of the DNA region. Such regulatory sequences include promoter regions, enhancer sequences and 3' regulatory sequences. A structural gene is a gene whose product is e.g. an enzyme, a structural protein, tRNA or rRNA. A regulatory gene is a gene which encodes a protein which regulates the expression (e.g. the transcription) of one or more structural or other regulatory genes.

For the purpose of this invention the expression of a gene (or of a DNA of the gene which encodes the RNA), such as a chimeric gene, means that the DNA region of the gene coding for the RNA is transcribed, under control of the promoter and other regulatory sequences of the gene, into a RNA which is biologically active i.e. which is either capable of interacting with another RNA, or which is capable of being translated into a biologically active polypeptide or protein.

The expression of most eucaryotic genes, including foreign (e.g. chimeric) genes, is regulated by combination of a minimal promoter element and one or more enhancer elements which bind to regulatory proteins. When a promoter directs expression of any DNA it is active. Depending on the amount of RNA produced by a promoter under a given set of conditions one can speak about low or high level of expression (or less or high activity of the promoter). With regard to the present invention a "high" level of expression of the male-sterility gene is interpreted as the level of expression in specific stamen cells whereby the production of fertile male gametes is prevented.

A minimal promoter element as used herein means a DNA that has the capacity to bind RNA polymerase and to inititate transcription. For any given gene the minimal promoter extends about 30-40, maximally 100, basepairs upstream from the transcription initiation site and generally includes the TATA box. An enhancer element is a regulatory element that is generally further upstream from the minimal promoter and that activates (or inhibits) transcription from the minimal promoter linked to it, with synthesis beginning at the normal start site. An enhancer is capable of binding transcription factors and can usually operate in both orientations and can function even when moved more than 1000 basepairs from the promoter and from either an upstream or a downstream position.

A promoter as used herein comprises a minimal promoter associated with one or more enhancer elements. For practical purposes a promoter and minimal promoter,

as used herein, may also comprise part of the DNA that is transcribed (e.g. the untranslated leader of a mRNA).

A transcriptional unit means a DNA segment that is transcribed into a continuous RNA from a promoter. For the purposes of this invention a transcriptional unit comprises the promoter.

A promoter which directs expression selectively in specific cells or tissues of a plant (e.g. stamen cells such as tapetum cells) is a promoter in which the enhancer elements operate to limit the transcription to specific cells or tissues in the plant and/or to specific stages of development of these specific cells or tissues, i.e. to enhance transcription in the specific cells or tissues at particular developmental stages and to inhibit transcription in all other cells or tissues or at other developmental stages. For all practical purposes such selective promoters are specific in activity and effect. Usually such selective promoters are identified by differential screening of mRNA libraries from different tissues (Sambrook et al., 1989, "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, and Ausubel et al, 1994, "Current Protocols in Molecular Biology", John Wiley & Sons). Although it is generally impossible to screen all tissues and all cells of a plant, promoters obtained in this way have been found to be useful to direct expression of heterologous DNA selectively in the same tissues in transgenic plants of the same and/or different plant species.

As used herein stamen cells will mean cells of at least one part of the male reproductive organ in a flower, in various stages of development, such as the filament, the anther, the tapetum, the anther cell wall, the pollen etc. A stamen-specific promoter is a promoter that is capable of directing expression (e.g. of barnase DNA) selectively in stamen cells (preferably including at least tapetum cells) at one or more stages in the development of the stamen to prevent the production of fertile pollen. It should be noted that a male-sterility gene comprising a pollen-specific promoter, i.e. a promoter that directs expression exclusively in microspores and/or pollen (i.e. after meiosis), when operably linked to a barnase DNA can only induce male-sterility in a plant when it is present in a homozygous form in the nuclear genome of that plant.

Non-stamen cells as used herein means all cells of a plant except the stamen cells (particularly the tapetum cells), especially those stamen cells in which the sterility promoter can direct expression of the barnase DNA.

The phenotype is the external appearance of the expression (or lack of expression) of a genotype i.e. of a gene or set of genes (e.g. male-sterility, presence of protein or RNA in specific plant tissues etc.).

As used herein, a genetic locus is a DNA (e.g. one or more genes) as defined with respect to its position in the nuclear genome, i.e. in a particular chromosome, of a plant. Two loci can be on different chromosomes and will segregate independently. Two loci can be located on the same chromosome and are then generally considered as being linked (unless sufficient recombination can occur between them).

An endogenous locus is a locus which is naturally present in a plant species. A foreign locus is a locus which is formed in the plant because of the introduction, e.g. by means of genetic transformation, of a foreign DNA. If a foreign DNA, which comprises two or more genes, is introduced in the plant genome this will generally be regarded as creating, in the plant genome, one foreign locus which comprises the two or more genes (although it can also be said that two or more closely linked loci are created).

In diploid plants, as in any other diploid organisms, two copies of a gene are present at any autosomal locus. Any gene can be present in the nuclear genome in several variant states designated as alleles. If two identical alleles are present at a locus that locus is designated as being homozygous, if different alleles are present, the locus is designated as being heterozygous. The allelic composition of a locus, or a set of loci, is the genotype. Any allele at a locus is generally represented by a separate symbol (e.g. M and m, S and -, - representing the absence of the gene). A foreign locus is generally characterized by the presence and/or absence of a foreign DNA. A dominant allele is generally represented by a capital letter and is usually associated with the presence of a biologically active gene product (e.g. a protein) and an observable phenotypic effect.

A plant can be genetically characterized by identification of the allelic state of at least one genetic locus.

The genotype of any given locus can be designated by the symbols for the two alleles that are present at the locus (e.g. M/m or m/m or S/-). The genotype of two unlinked loci can be represented as a sequence of the genotype of each locus (e.g. S/S,R/-)

Foreign male-sterility loci are those in which the allele responsible for male sterility is a foreign DNA sequence S which comprises the male-sterility gene which when expressed in cells of the plant renders the plant male-sterile without otherwise substantially affecting the growth and development of the plant.

The male-sterility locus preferably also comprises in the same genetic locus at least one marker gene T which comprises at least:

t1) a marker DNA encoding a marker RNA, protein or polypeptide which, when present at least in a specific tissue or specific cells of the plant, renders the plant easily separable from other plants which do not contain the marker RNA, protein or polypeptide encoded by the marker DNA at least in the specific tissue or specific cells, and,

t2) a marker promoter capable of directing expression of the marker DNA at least in the specific tissue or specific cells: the marker DNA being in the same transcriptional unit as, and under the control of, the marker promoter.

Such male-sterility gene is always a dominant allele at such a foreign male-sterility locus. The recessive allele corresponds to the absence of the male-sterility gene in the nuclear genome of the plant.

Male-sterility DNAs and sterility promoters that can be used in the male-sterility genes of this invention have been described before (EP 0,344,029 and EP 0,412,911). For the purpose of this invention the expression of the male-sterility gene in a plant cell should be able to be inhibited or repressed for instance by means of expression of a suitable fertility-restorer gene in the same plant cell. In this regard a particular useful male-sterility DNA codes for barnase (Hartley, J.Mol. Biol. 1988 202:913). The sterility promoter can be any promoter but it should at least be active in stamen cells, particularly tapetum cells. Particularly useful sterility promoters are promoters that are selectively active in stamen cells, such as the tapetum-specific promoters of the TA29 gene of Nicotiana tabacum (EP 0,344,029) which can be used

in tobacco, oilseed rape and other Brassica species, cichory, corn, rice, wheat and other plant species; the PT72, the PT42 and PE1 promoters from rice which can be used in rice, corn, wheat, and other plant species (WO 92/13956) ; the PCA55 promoter from corn which can be used in corn, rice, wheat and other plant species (WO 92/13957); and the A9 promoter of a tapetum-specific gene of Arabidopsis thaliana (Paul et al., 1992, Plant Mol. Biol. 19:611-922).

It has been found that stamen-specific promoters, such as PTA29, operably linked to a suitable sterility DNA, such as the barnase DNA, can be used in a variety of plant species to induce male-sterility. Indeed, by transformation of plants with such male-sterility genes, male-sterile lines with high agronomic value have been obtained in many plant species. Apparently, the stamen-specific promoters, for all practical purposes, substantially retain their spatial and temporal specificity. However, not all individual transformed plants can be developed into lines with good agronomical performance. Indeed some plants show undesired phenotypic effects which can be due to somaclonal variation and/or 'position effects'. It is believed that at least part of this variation is due to the regulating effects of native (i.e. endogenous) enhancer elements in the plant genome that surround the integrated male-sterility gene in the transgenic plants. Such enhancer sequences, and consequently their effects on the expression of the male-sterility gene, differ depending on the place of integration of the male-sterility gene. This can result, in some transformants, in low-level (often even undetectable) expression of the sterility DNA (e.g. barnase DNA) in tissues other than the stamen cells, e.g. in cells during tissue culture or in somatic cells of the plants or seeds.

In this regard, this invention is based on the observation that, under some circumstances, a chimeric gene such as the barstar gene, introduced together with a male-sterility gene such as a gene comprising barnase DNA can decrease the between-transformant variability in expression of the male-sterility gene, and of its resulting phenotype, and can increase the frequency of transformants having good agronomical performance. For the purposes of this invention it is therefore preferred that the sterility DNA is the barnase DNA while the coregulating DNA is the barstar DNA.

For the purposes of this invention barnase DNA means a DNA coding for the ribonuclease of Bacillus amyloliquefaciens with the amino acid sequence as described by Hartley, 1988, J.Mol.Biol. 202:913-915 (barnase s.s.) or any variants thereof which have ribonuclease activity and are capable of being inactivated by barstar. In this regard one of such variants of barnase s.s. has been found to be encoded by the DNA of Bacillus intermedius which encodes a ribonuclease (binase) which has 84% identity at the amino acid level with barnase s.s. (Schulga et al, 1992, NAR 20:2375; see also Guillet et all, 1993, Structure 1:165-177). Preferably, the barnase variants retain at least 10% particularly at least 50% of the activity of barnase s.s. as measured under standard conditions (Fitzgerald and Hartley. 1993, Anal. Biochem. 214:544-547; Hartley et al, 1993, Biochemistry 32:5978-5984).

For the purposes of this invention barstar DNA means a DNA coding for an inhibitor of the barnase ribonuclease of Bacillus amyloliquefaciens as described by Hartley, 1988, J.Mol.Biol. 202:913-915 (barstar s.s.) or any variants thereof which are capable of inhibiting barnase s.s. In this regard one of such variants has been found to be encoded by the DNA Bacillus intermedius which encodes binstar (Guillet et al, 1993, Structure 1:165-177). Preferably the barstar variants are capable of inhibiting at least 90% of barnase activity, particularly at least 50% of barnase activity, in an equimolar mixture of the barstar variant and barnase in standard condition (Hartley et al, 1993, Biochemistry 32:5978-5984).

However, any DNA coding for a ribonuclease can be used as sterility DNA in this invention provided a DNA coding for protein inhibitor of that ribonuclease can be obtained. Examples of such RNAses and corresponding inhibitors are for instance listed in Guillet et al, 1993, Structure 1:165-177. Another example of such a ribonuclease is the RNAse Sa or sarnase of Streptomyces aureofaciens (Shlyapnikov et al, 1986, FEBS Letters 209:335-339; Homerova et al, 1992, Gene 119:147-148). An inhibitor of RNAse Sa is known (Mucha et al, 1983, Biologia 38:1177-1184).

Of course, any sterility DNA coding for a RNA, protein or polypeptide and its corresponding coregulating DNA coding for a coregulating RNA, protein or polypeptide which, when expressed in the same plant cell as the sterility DNA is

capable of preventing expression of the sterility DNA or the activity of the sterility RNA, protein or polypeptide can be used. In this regard DNAs that are described as fertility restorer DNAs in EP 0,412,911 can be used as coregulating DNAs of this invention in combination with their corresponding sterility DNAs which are also described in EP 0,412,911.

The promoter in the coregulating gene (the "coregulating promoter") of this invention is preferably capable of driving expression of the coregulating DNA (e.g. the barstar DNA) in a variety of cells and tissues, preferably all cells and tissues, of the plant to counteract the undesired effects of possible low level expression of the male-sterility gene (e.g. comprising the barnase DNA). In this regard, the promoter can also drive expression in those stamen cells in which the sterility promoter drives expression of barnase (as an example of a sterility DNA) and which are killed by the biological activity of the barnase which prevents the production of fertile male gametes. Of course in such stamen cells the activity of the sterility promoter and the coregulating promoter should be such that for instance the amount of produced barnase in such stamen cells is higher than that of the produced barstar at least during a period in stamen development. In this regard it is preferred that the coregulating promoter is not active in the same stamen cells as the sterility promoter. However, outside the stamen cells (e.g. the tapetum) in which the sterility promoter drives expression of the barnase DNA, the coregulating promoter may be active at any level. If the coregulating promoter is active in the same stamen cells as the sterility promoter (but so that sufficient barnase is still produced in the stamen cells to render the plant male-sterile) this can have the added advantage that the restoration of male fertility in the progeny of these male-sterile plants after crossing with restorer plants containing a fertility-restorer gene (e.g. comprising the barstar DNA under control of a stamen-specific promoter), is generally easier due to the fact that the amount of barnase in the stamen cells is already reduced due to expression of the coregulating gene.

Preferably the coregulating promoter is a promoter operable in plant cells and such many promoters can be used in this invention (see e.g. Fig. 1, A). In a preferred embodiment the 35S promoter ("P35S") of the Cauliflower Mosaic virus is used. This

is a family of promoters that are generally known as constitutive promoters but that appear to be relatively less active in anther cells, particularly in tapetum cells. Surprisingly it was found that the activity of the P35S is sufficiently low in tapetum cells and that it can be used together with a male-sterility gene comprising a tapetum-specific promoter. Even more surprisingly it was found that the use of the P35S as coregulating promoter was particularly effective in rice, especially when PT72 and pE1 are used as sterility promoters, and in corn, especially when PCA55 or PTA29 are used as sterility promoters.

Suitable P35S promoters can be obtained from the Cauliflower Mosaic Virus ("CaMV") isolates CM1841 (Gardner et al (1981) Nucl. Acids. Res. 9:2871) and CabbB-S (Franck et al (1980) Cell, 21:285) (the "35S2 promoter" or "P35S2"), from the CaMV isolate CabbB-JI (Hull and Howell (1978) Virology 86:482) (the "35S3 promoter" or "P35S3"). P35S3 differs from P35S2 in its sequence (the sequence of P35S3 is disclosed in European patent publication ("EP") 359617) and in its greater activity in transgenic plants (Harpster et al (1988) Mol. Gen. Genet. 212:182).

Of course other known constitutive promoters can be used as coregulating promoter. For instance the promoter of the nopaline synthase gene of Agrobacterium T-DNA ("Pnos") is known to drive low-level expression in a constitutive way in plants. It is believed that Pnos is particularly effective as coregulating promoter in dicot plants, such as Brassica species, e.g. Brassica napus.

Other suitable constitutive promoters that can be used as coregulating promoters are the TR1' and the TR2' promoters (resp. "PTR1" and "PTR2") which drive the expression of the 1' and 2' genes, respectively, of the T-DNA of Agrobacterium (Velten et al (1984) EMBO J. 3:2723), and are wound-induced promoters that are only weakly active in the uninduced state.

Suitable organ-specific, tissue-specific and/or inducible foreign promoters can also be used as coregulating promoters such as the promoters of the small subunit genes (such as the 1A gene) of 1,5-ribulose bisphosphate carboxylase of Arabidopsis thaliana (the "ssu" promoter) which are light inducible promoters (Krebbers et al (1988) Plant Mol. Biol. 11:745) active primarily in photosynthetic tissue; and the seed-specific promoters of, for example, Arabidopsis thaliana

(Krebbers et al (1988) Plant Physiol. 87:859), and the promoter of the Kunitz trypsine inhibitor gene (Jofuku and Goldberg, 1989, The Plant Cell 1:1079-1093).

In another preferred embodiment of this invention (see e.g. Fig. 1, B) the coregulating promoter comprises a minimal promoter element which can be derived from any promoter that can be expressed in plant cells including constitutive promoters (P35S, Pnos), tissue-specific promoters (PTA29, PCA55, PT72, PE1, PT42), or inducible promoters (e.g. PTR1, PTR2, Pssu). Such minimal promoter element is the sequence comprising about 30-50, maximally about 100 basepairs upstream from the transcription start site and which contains the TATA box.

Such a minimal promoter element can be used in the coregulating gene of this invention to direct low-level transciption of the barstar DNA in non-stamen cells.

In addition, the position effects in transgene expression can now be used to good effect. Indeed, the plant genomic DNA that is adjacent to the foreign DNA (or transgene) may comprise additional sequences, such as enhancer sequences, that are capable of regulating the minimal promoter to enhance transcription of the barstar DNA in a variety of plant cells. In this regard it is preferred that the coregulating gene is provided in a transforming DNA in such a way that especially upstream sequences are brought in optimal position to the minimal promoter. In this regard it is preferred that the coregulating gene is present at the extreme ends of the foreign DNA (e.g. the T-DNA).

The coregulating gene may even be lacking sequences required for being transcribed in a plant cell. For instance the coregulating gene may only comprise the coregulating DNA or it may comprise the coregulating DNA with upstream sequences that are not capable of directing expression of the coregulating DNA in plant cells. Thus the coregulating gene may lack a suitable promoter or it may comprise a bacterial promoter. (e.g. the native promoter of the barstar gene in B amyloliquefaciens or the tac promoter) (see e.g. Fig. 1, C). However, in this instance, it is preferred that the coregulating gene is present at the extreme ends of the foreign DNA used for plant transformation (e.g. the T-DNA) in such an orientation that the translation initiation codon of the coregulating DNA is closest to one of the ends of the foreign DNA. Indeed, it is believed that this orientation increases the probability

that the coregulating gene, when inserted in the plant genome, is placed under control of (i.e. has "captured") suitable promoter (e.g. minimal promoters) and/or enhancer sequences in the adjacent plant genomic DNA to enable the more or less constitutive expression of the coregulating DNA such as the barstar DNA. Because it is unlikely that the plant promoter and/or enhancer sequences will be optimally positioned with respect to the barstar DNA, it is expected that the level of any expression of the barstar DNA will be very low, as desired in many cases.

The male-sterility gene and the coregulating gene are preferably inserted in the plant genome as a single transforming DNA. Therefore both genes should preferably be present on the same vector or should be part of the same T-DNA.

However, both genes could also be present on separate DNAs which are both used for transformation. In such "cotransformation" it has been found that both DNAs are likely to be integrated in the same genetic locus of the plant genome, although there is of course a probability that both genes are integrated at different locations in the plant genome. In this respect the foreign DNA used for transformation of the nuclear genome of a plant cell need not be a single DNA molecule but can be multiple DNA molecules. For the purpose of the present invention it is however preferred that the male-sterility gene and the coregulating gene be integrated in the same locus in the plant nuclear genome.

However, if the coregulating gene is useful to counteract the low level expression of the male-sterility gene in tissue culture, its presence might not be required in the mature plants and their progeny. If the plants are transformed by cotransformation, and if the male-sterility gene and coregulating gene are integrated at different locations in the plant genome, then both genes will segregate in the progeny and the coregulating gene can hereby be removed from the transformed plant line.

The male sterile plants of this invention can be crossed with male-fertile parent plants, particularly a male-fertile restorer plant containing a suitable fertility restorer gene (see e.g. EP 0,412,911)

Marker DNAs and marker promoters that can be used in the marker gene as used in this invention are also well known (EP 0,344,029; EP 0,412,911).

Foreign DNA such as the male-sterility gene, the fertility-restorer gene, the coregulating gene, or the marker gene preferably also are provided with suitable 3' transcription regulation sequences and polyadenylation signals, downstream (i.e. 3') from their coding sequence i.e. respectively the fertility-restorer DNA, the male-sterility DNA, the coregulating DNA or the marker DNA. In this regard either foreign or endogenous transcription 3' end formation and polyadenylation signals suitable for obtaining expression of the chimeric gene can be used. For example, the foreign 3' untranslated ends of genes, such as gene 7 (Velten and Schell (1985) Nucl. Acids Res. 13:6998), the octopine synthase gene (De Greve et al., 1982, J.Mol. Appl. Genet. 1:499; Gielen et al (1983) EMBO J. 3:835; Ingelbrecht et al., 1989, The Plant Cell 1:671) and the nopaline synthase gene of the T-DNA region of Agrobacterium tumefaciens Ti-plasmid (De Picker et al., 1982, J.Mol. Appl. Genet. 1:561), or the chalcone synthase gene (Sommer and Saedler, 1986, Mol.Gen.Genet. 202:429-434), or the CaMV 19S/35S transcription unit (Mogen et al., 1990, The Plant Cell 2:1261-1272) can be used.

The fertility-restorer gene, the male-sterility gene, the coregulating gene or the marker gene in accordance with the present invention are generally foreign DNAs, preferably foreign chimeric DNA. In this regard "foreign" and "chimeric" with regard to such DNAs have the same meanings as described in EP 0,344,029 and EP 0,412,911.

The cell of a plant, particularly a plant capable of being infected with Agrobacterium such as most dicotyledonous plants (e.g. Brassica napus) and some monocotyledonous plants, can be transformed using a vector that is a disarmed Ti-plasmid containing the male-sterility gene and/or the coregulating gene (preferably both) and carried by Agrobacterium. This transformation can be carried out using the procedures described, for example, in EP 0,116,718 and EP 0,270,822. Preferred Ti-plasmid vectors contain the foreign DNA between the border sequences, or at least located to the left of the right border sequence, of the T-DNA of the Ti-plasmid. Of course, other types of vectors can be used to transform the plant cell, using procedures such as direct gene transfer (as described, for example, in EP 0,233,247), pollen mediated transformation (as described, for example, in EP

0,270,356, PCT patent publication "WO" 85/01856, and US patent 4,684,611), plant RNA virus-mediated transformation (as described, for example, in EP 0,067,553 and US patent 4,407,956) and liposome-mediated transformation (as described, for example, in US patent 4,536,475). Cells of monocotyledonous plants such as the major cereals including corn, rice, wheat, barley, and rye, can be transformed (e.g. by electroporation) using wounded or enzyme-degraded intact tissues capable of forming compact embryogenic callus (such as immature embryos in corn), or the embryogenic callus (such as type I callus in corn) obtained thereof, as described in WO 92/09696. In case the plant to be transformed is corn, other recently developed methods can also be used such as, for example, the method described for certain lines of corn by Fromm et al., 1990, Bio/Technology 8:833; Gordon-Kamm et al., 1990, Bio/Technology 2:603 and Gould et al., 1991, Plant Physiol. 95:426. In case the plant to be transformed is rice, recently developed methods can also be used such as, for example, the method described for certain lines of rice by Shimamoto et al., 1989, Nature 338:274; Datta et al., 1990, Bio/Technology 8:736; and Hayashimoto et al., 1990, Plant Physiol. 93:857.

The transformed cell can be regenerated into a mature plant and the resulting transformed plant can be used in a conventional breeding scheme to produce more transformed plants with the same characteristics or to introduce the male-sterility gene, the coregulating gene (or both), in other varieties of the same related plant species. Seeds obtained from the transformed plants contain the chimeric gene(s) of this invention as a stable genomic insert. Thus the male-sterility gene, and/or the coregulating gene of this invention when introduced into a particular line of a plant species can always be introduced into any other line by backcrossing.

The present invention thus provides a method to obtain male-sterile plants whereby the frequency of obtaining, from transformation, male-sterile plants with good agronomic performance is increased. This is because the coregulating gene is expressed in non-stamen cells. In this regard the presence of the coregulating gene may counteract a number of phenomena such as:

- low-level expression of the male-sterility gene in some transformed plant cells in tissue culture, including regeneration prior to normal plant development. Indeed

such tissue culture cells have a physiology and metabolism and patterns of gene regulation which may be different from that of any differentiated cell in a plant or seed. Since the sterility promoter is generally selected on the basis of its natural activity in the plant or seed, position effects are perhaps expected to be more pronounced to activate the promoter in tissue culture cells. When direct gene transfer is used an additional phenomenon may occur. Indeed in such transformation method a large amount of DNA is delivered to any recipient cell. If gene repression should be an active process, which requires for instance DNA methylation or repressor protein binding, the repression mechanism may become temporarily overloaded, and the delivered DNA may be expressed for a short period of time. It can be seen that the coregulating gene can thus increase the general transformation efficiency.

- low-level expression of the sterility DNA (e.g. the barnase DNA) in specific non-stamen cells of the primary transformants and/or particularly the progeny plants obtained thereof. Such low level expression can be due to several factors many of which are largely unknown:
  - activation of the stamen-specific promoter by elements in the vector used for transformation,
  - position effects as outlined above. Such effects may possibly be more pronounced in plants with a small genome and little repetitive DNA, such as rice.
  - rearrangements in additional copies of the transgene. This is most likely to occur in transformation by direct gene transfer in which multiple copies of the transforming DNA are often integrated at the same genetic locus in the plant genome with subsequent rearrangements of some of the copies. During such rearrangements, a DNA containing barnase DNA could be inadvertently be placed under control of a promoter present in the transforming DNA (e.g. the P35S promoter) or in the adjacent plant genomic DNA.

Whatever the reason, the use in plant transformation of a coregulating gene of this invention combined with a corresponding male-sterility gene will generally result in a higher frequency of male-sterile transgenic plants with good agricultural performance.

It will also be appreciated that the coregulating genes of the invention will be useful in combination with a pseudo male-sterility gene which comprises a male-sterility DNA that is under control of promoters that are not entirely stamen-specific, but that also are known to direct expression in some other tissue(s) outside the stamen (e;g. seeds). In this regard the coregulating promoter should be a promoter that is active in these some other tissue(s) to a sufficient level to counter the expression of the pseudo male-sterility gene but that is does not prevent the pseudo male-sterility gene" to be expressed in stamen cells (e.g. tapetum, anther-epidermal cells). In this regard the pseudo male-sterility gene and the coregulating gene together will be equivalent to a male-sterility gene which comprises a true stamen-specific promoter.

As already indicated, the invention allows the generation of a higher number of male-sterile plants with good agronomical performance. In such plants the male-sterility gene will be genetically stable, i.e. the gene should be inherited and all plants comprising the gene should be male-sterile. Nevertheless it may not be absolutely required that all seeds that contain the gene are viable (i.e. will grow into normal mature plants). It is generally sufficient that from each male-sterile plant viable seeds that have inherited the male-sterility gene can be obtained.

Preferably the performance of the male-sterility gene (i.e. its phenotypic expression) should also be independent on genetic background so that the gene can be readily introduced in other lines through backcrossing.

It is generally also required that the male-sterility genotype is environmentally stable and that the phenotype will be independent of the various environmental conditions that can occur in the area and period in which the plants will be grown. Such environmental stability is usually demonstrated by performing field trials with the male-sterile plants in 3 or 4 different locations.

It is generally also desired that the male-sterility genotype has no significant negative effects on agronomically important characteristics and on plant development. Nevertheless this will depend not only on the performance of the male-sterile parent line, but also of the performance of the hybrid obtained from that parent line. Indeed, these negative effects can in some circumstances be compensated by significant advantages in the hybrid.

Finally, in plant species where restoration of fertility is required, either in maintenance of the male-sterile line, or in hybrid seed production, the male-sterility genotypes should be restorable by at least one fertility restorer gene.

Unless otherwise indicated all experimental procedures for manipulating recombinant DNA were carried out by the standardized procedures described in Sambrook et al., 1989, "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, and Ausubel et al, 1994, "Current Protocols in Molecular Biology", John Wiley & Sons, Vols 1 and 2.

The polymerase chain reactions ("PCR") were used to clone and/or amplify DNA fragments. PCR with overlap extension was used in order to construct chimeric genes (Horton et al, 1989, Gene 77:61-68; Ho et al, 1989, Gene 77:51-59).

All PCR reactions were performed under conventional conditions using the Vent™ polymerase (Cat. No. 254L - Biolabs New England, Beverley, MA 01915, U.S.A.) isolated from Thermococcus litoralis (Neuner et al., 1990, Arch.Microbiol. 153:205-207). Oligonucleotides were designed according to known rules as outlined for example by Kramer and Fritz (1987, Methods in Enzymology 154:350), and synthesized by the phosphoramidite method (Beaucage and Caruthers, 1981, Tetrahedron Letters 22:1859) on an Applied Biosystems 380A DNA synthesizer (Applied Biosystems B.V., Maarssen, Netherlands).

In the description and in the following examples, reference is made to the following figure and sequence listing:

FIGURES

Figure 1 : Schematic presentation of three examples of foreign DNA of this invention:

Pster : sterility promoter

barnase : region coding for barnase (as example of a sterility DNA)

3'end : 3' untranslated region of a gene (e.g. 3'nos, 3'g7)

Pcoreg : coregulating promoter (e.g. P35S, Pnos)

barstar : region coding for barstar (as example of coregulating DNA)

Pmin : minimal plant promoter

SEQUENCE LISTING

SEQ ID NO 1: pTS174

SEQ ID NO 2: pTS88 HindIII-EcoRI

SEQ ID NO 3: pVE136 - EcoRI-HindIII

SEQ ID NO 4: T-DNA of pTCO113

Examples

Example 1 : Coregulating genes in rice

Compact embryogenic callus from rice cultivar Kochihibiki was obtained and transformation of callus cells by electroporation was achieved using the procedures as described in WO 92/096096, particularly in Example 9, except that the transforming DNA consisted of either plasmid pTS174 or plasmids pTS174 and pTS88 in equimolar amounts or more preferably in a 1:3 molar ratio. Prior to transformation, pTS174 and pTS88 were preferably linearized by digestion with

appropriate restriction enzymes. All tissue culture steps were also carried out as decribed in WO 92/09696, example 9.

pTS174 is a pUC19 derived plasmid containing, in its polylinker, the barnase DNA under control of the PE1 promoter (PE1-barnase-3'nos) and the bar gene under control of a 35S promoter (P35S-bar-3'g7). The sequence of pTS174 is given in SEQ ID No 1. pTS88 is a pGEM2 derived plasmid containing, between the HindIII and EcoRI sites of its polylinker, the barstar DNA under control of a 35S promoter (P35S-barstar-3'g7). The sequence of the HindIII-EcoRI fragment of pTS88 is given in SEQ ID No 2. All chimeric genes comprising barstar, barnase, or bar also contained suitable 3' untranslated regions (e.g. of the nopaline synthase gene (3'nos) and gene 7 (3'g7) of Agrobacterium T-DNA).

Plasmids containing a fertility restorer gene comprising the barstar DNA under control of stamen-specific promoter of rice (PE1-barstar), and a herbicide resistance gene comprising the bar gene under control of the 35S promoter (P35S-bar) chimeric genes were used as control.

The results of the transformation experiments is presented in Table 1. In transformation experiments with pTS174 only one normal male-sterile line could be recovered from 48 electroporation cuvettes. In transformation experiments with PTS174 + pTS88, 7 normal male sterile lines could be recovered from 40 cuvettes. Each cuvette contained about 50 callus pieces (approximately 1-2 mm in diameter) of tissue fragments.

In this regard a normal male-sterile plant is understood to be a male-sterile rice plant (i.e. with small, white anthers that do not contain pollen) that is otherwise completely normal (e.g. is female-fertile) and that transmits the male-sterility phenotype to its progeny in accordance with normal Mendelian segregation of the chimeric barnase gene.

From table 1 it is also clear that the advantage of using pTS174 + pTS88 over using pTS174 alone resides in the number of normal regenerated shoots that can be recovered on selective regeneration medium thus attesting to the fact that the P35S-barstar gene affects mainly cells in tissue culture. In this regard it is also important to note that plants containing both the P35S-barstar-3'g7 and the PE1-barnase-3'nos

chimeric genes are male-sterile attesting to the fact that the P35S promoter is not active (or less active than the PE1 promoter) in specific stamen cells (particularly tapetum cells) of rice plants.

Example 2 : Coregulating genes in corn.

Maize plants of lines H99, Pa91 and (Pa91xH99)xH99 ((PxH)xH) were grown in the greenhouse. Type I callus was initiated from immature zygotic embryos of 1 to 1.5 mm in size, which were excised from ears 10 to 14 days after pollination and then plated on MahIVII callus initiation medium (D'Halluin et al, 1992, The Plant Cell 4:1495-1505). Embryogenic callus was removed from the scutella of the embryos and subcultured every 2 to 3 weeks on Mah1VII substrate. Pieces of embryogenic tissue (about 1 to 1.5 mm in diameter) were isolated from actively growing embryogenic callus cultures and were placed on a plate with MahIVII substrate supplemented with 0.2 M mannitol and 0.2 M sorbitol for osmotic pretreatment for 4 hours before bombardment (Vain et al, 1993, Plant Cell Reports 12:84-88). A total amount of about 250 mg of tissue per plate was used in the bombardment experiments. DNA was bombarded into the tissue using the PDS-1000/He Biolistics® device (Bio-Rad). Microcarrier preparation and coating of DNA onto the microcarriers was essentially as described by Sanford et al (1993, In Wu, R. (Ed.). Meth. Enzymol. 217:483-509). The particle bombardment parameters were: target distance: 6 to 9 cm; bombardment pressures: 1100 to 1500 psi; gap distance: 1/4 inches; macrocarrier flight distance: 11 mm. DNA was either linear or circular. The bombarded tissue was removed from the high osmotic medium (between 0 to 24 hours after bombardment) and transferred to selective maintenance medium without caseïne hydrolysate and proline, but containing 10 to 20 mg/l BASTA. The embryogenic callus was subcultured every 2 to 3 weeks for a total period of 6 to 8 weeks and was then transferred to MS medium (Murashige and Skoog, 1962, Physiol. Plant. 15:473-497) containing 3% sucrose, 10-20 mg/l BASTA, and 5 mg/l BAP (for lines H99) or 5 mg/l zeatine (for lines Pa91 or (PxH)xH). The embryogenic tissue was subcultured twice on substrate containing the appropriate cytokinin. Small regenerating plants were

recovered and transferred to MS medium without hormones, but containing 6% sucrose and 10-20 mg/l BASTA. Further developing shoots were transferred to half-strength MS medium with 1.5 % sucrose for further elongation. The resulting plantlets were then transferred to soil in the greenhouse. It was found that after the transformation step, the concentration of BASTA in the culture medium could be reduced down to 2 mg/l.

The following DNA was used. In one set of experiments callus was transformed with plasmid pVE136 which is a pUC19 derived plasmid containing, between the EcoRI and HindIII sites of its polylinker, the barnase DNA under control of the PCA55 promoter (PCA55-barnase-3'nos) and a chimeric P35S-bar-3'nos gene. The sequence of the EcoRI-HindIII fragment of pVE136 is given in SEQ ID. No. 3. In other experiments callus was bombarded with an equimolar mixture of pVE136 and pTS88. pTS88 is the plasmid described in Example 1. In control experiments callus was bombarded with plasmid pDE110 which is a plasmid containing only the P35S-bar-3'nos chimeric gene and is described in WO 92/29696.

The results of the transformation experiments are presented in Table 2. In transformation experiments with pVE136 + pTS88 the number of PAT positive plants, relative to the starting material, is almost twice that obtained in experiments using pVE136 alone.

It is important to note that corn plants containing both the P35S-barstar-3'g7 and the PCA55-barnase-3'nos chimeric genes are male-sterile attesting to the fact that the P35S promoter is not active (or less active than the PCA55 promoter) in stamen cells of corn plants.

Example 3 : Coregulating genes in oilseed rape

Oilseed rape plants (Brassica napus - both spring and winter varieties) were transformed with plasmid pTCO113 using the Agrobacterium mediated transformation procedure essentially as decribed by De Block et al, 1989, Plant Physiol. 91:694-701.

Plasmid pTCO113 is a intermediate cloning vector (T-DNA vector) containing between Agrobacterium T-DNA borders the following genes:

- the bar gene under control of the PSSU promoter
- the barnase gene under control of the PTA29 promoter
- the barstar gene under control of the Pnos promoter.

The sequence of the T-DNA of pTCO113 is presented in SEQ ID. No 4.

Transformation efficiency with pTCO113 was observed to be equal to that obtained with pTHW107 which is a T-DNA vector that is identical to pTCO113 but lacks the Pnos-barstar gene (the nucleotide sequence of pTHW107 is identical to that of SEQ ID No. 4 except that it lacks the nucleotide region 4917-5834).

Oilseed rape plants transformed with pTC0113 were observed to be male-sterile. More precisely, of 31 spring oilseed rape plants regenerated after transformation with pTCO113, 27 plants (87%) were shown to be male-sterile. Of 22 spring oilseed rape plants regenerated after transformation with pTHW107, 20 plants (91%) were shown to be male-sterile (Table 3).

Seeds harvested from male-sterile TO plants pollinated by untransformed male-fertile plants, were grown into T1 plants in the greenhouse. 50% of the plants of each T1 line are expected to carry the male-sterility gene. Plants were analyzed at the time that 50%, respectively 100% of the plants had started flowering. It was observed that plants transformed with pTCO113 have a smaller delay of flowering as compared to plants transformed with pTHW107. This was measured by the ratio of male-fertile (F)/male-sterile (S) plants at the moment that 50% of the plants had started to flower

(Table 3). When all plants flowered, the ratio F/S was 54/46 for both pTCO113 and pTHW107 plants.

The seeds harvested from male-sterile T1 plants of different lines, pollinated by untransformed male-fertile plants, were sown in the field and analyzed with respect to the segregation of the male-sterility genes in the T2 progeny plants. Only 2 out of 7 tested pTHW107 lines, but no less than 12 out of 14 tested pTCO113 lines, showed a normal 1:1 Mendelian segregation (X^2=6.86, $p<0.01$) (Table 3).
It can therefore be conducted that in transformation experiments with pTCO113 a higher percentage of good male-sterile plants was obtained.

TABLE 1

DNA	Total Nr of cuvettes	Regenerants			
	(Nr of experiments)	PAT+[1] normal shoots	PCR+[2]	male-sterility phenotype [3]	progeny analysis[4]
pTS174	48 (9)	1	1/1	1/1	1/1
pTS174 + pTS88	40 (8)	33	24/33	9/24	7/9
FR constructs	23(9)	23	-	-	-

1) Total number of shoots regenerated on PPT (i.e. selective) medium that appeared phenotypically normal
2) Number of PCR+ plants/Number of analyzed PAT+ plants. PCR+ for barnase or barnase/barstar.
3) Number of male-sterile but otherwise normal plants/number of analyzed barnase PCR+ plants
4) Number of phenotypically normal male-sterile plants with good segregation of male-sterile phenotype in progeny/Nr of analyzed male-sterile plants

TABLE 2

DNA	Total Nr of bombarded plates	Regenerants			Progeny analysis
		PAT+[1]	PCR+[2] barnase	male-sterility phenotype [3]	
pVE136	118	68[5]	34/62	27/34	7/16
pVE136+pTS88	131	141	82/125	64/82	17/34
pDE110[6]	65	125	-	-	

1) Total number of PAT+ regenerants recovered from all transformation experiments.
2) Number of PCR+ plants/Number of analyzed PAT+ plants
3) Number of male-sterile plants/number of analyzed barnasePCR+ plants
4) Number of male-sterile plants with good segregation (1:1) of male-sterile phenotype in progeny/Nr of analyzed male-sterile plants
5) Number of selected calli was significantly less when compared to calli of transformation experiments containing P35S-barstar
6) Cotransformation experiments using pDE110 in combination with plasmids not comprising cytotoxic genes.

TABLE 3

	T0[1]	T1[2]		T2
		F/S at 50% Flowering	F/S at 100% Flowering	Progeny Analysis[3]
pTHW107	20/22	72% / 28%	54% / 46%	2 / 7
pTCO113	27/31	62% / 38%	54% / 46%	12 / 14

1) T0 : Number of transformed plants that were male-sterile/number of Basta-tolerant plants regenerated after transformation.

2) T1 : percentage of T1 plants with male-fertile flowers (F) / percentage of T1 plants with male-sterile flowers (S) at a time that 50%, respectively 100%, of the T1 plants started to flower. Data from different lines (18 pTHW107 and 17 pTCO113 lines respectively) were pooled.

3) T2: Number of T1 lines that have a normal 1:1 segregation of the male-sterility gene / total numbers of T1 lines that were examined in the field.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: PLANT GENETIC SYSTEMS N.V.
(B) STREET: Plateaustraat 22
(C) CITY: Ghent
(E) COUNTRY: Belgium
(F) POSTAL CODE (ZIP): 9000
(G) TELEPHONE: 32 9 235 84 58
(H) TELEFAX: 32 9 224 06 94
(I) TELEX: 11.361 Pgsgen

(ii) TITLE OF INVENTION: Method to obtain male sterile plants

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6548 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: plasmid pTS174

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..2003
(D) OTHER INFORMATION:/label= vector
/note= "pUC19 derived vector sequences"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (2019..2283)
(D) OTHER INFORMATION:/label= 3'nos
/note= "region containing polyadenylation signal of nopaline
synthase gene of Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (2284..2624)
(D) OTHER INFORMATION:/label= barnase
/note= "region coding for barnase of Bacillus amyloliquefaciens"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (2625..4313)
(D) OTHER INFORMATION:/label= PE1
/note= "promoter of the stamen-specific E1 gene of rice"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:4336..5710
(D) OTHER INFORMATION:/label= P35S
/note= "35S promoter of Cauliflower Mosaic Virus"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:5711..6262
(D) OTHER INFORMATION:/label= bar
/note= "region coding for phosphinothricin acetyl transferase"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:6263..6496
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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ATATTTCTTT CAAGATGGGA ATTAACATCT ACAAATTGCC TTTTCTTATC GACCATGTAC 6540

GTATCGCG 6548

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1303 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HindIII-EcoRI fragment of pTS88

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..35
(D) OTHER INFORMATION:/label= pGEM2
/note= "polylinker of pGEM2"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:36..694
(D) OTHER INFORMATION:/label= P35S
/note= "35S promoter of Cauliflower Mosaic Virus strain CM1841"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:695..967
(D) OTHER INFORMATION:/label= barstar
/note= "region coding for barstar of Bacillus amyloliquefaciens"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:968..1287
(D) OTHER INFORMATION:/label= 3'g7
/note= "region containing polyadenylation signal of gene 7 of
Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -

(B) LOCATION:1288..1303
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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3658 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: EcoRI-HindIII fragment of pVE136

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..26
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synthase gene of Agrobacterium T-DNA"

(ix) FEATURE:
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(D) OTHER INFORMATION:/label= bar
/note= "region coding for phosphinothricin acetyl transferase"

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(B) LOCATION:3341..3623
(D) OTHER INFORMATION:/label= 3'nos
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synthase gene of Agrobacterium T-DNA"

(ix) FEATURE:
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(B) LOCATION:3624..3658
(D) OTHER INFORMATION:/label= pUC19
/note= "polylinker of pUC19"

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CATCTATGTT ACTAGATCGG GAAGATCCTC TAGAGTCGAC CTGCAGGCAT GCAAGCTT 3658

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5864 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: T-DNA of plasmid pTCO113

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (1..25)
(D) OTHER INFORMATION:/label= RB
/note= "right border of Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (98..330)
(D) OTHER INFORMATION:/label= 3'g7
/note= "region containing polyadenylation signal of gene 7 of
Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (331..882)
(D) OTHER INFORMATION:/label= bar
/note= "region coding for phosphinothricin acetyl transferase"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (883..2608)
(D) OTHER INFORMATION:/label= Pssu
/note= "promoter of small subunit gene of Rubisco of Arabidopsis"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (2659..3031)
(D) OTHER INFORMATION:/label= 3'nos
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synthase gene of Agrobacterium T-DNA"

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:complement (3032..3367)

(D) OTHER INFORMATION:/label= barnase
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(ix) FEATURE:
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(B) LOCATION:complement (3368..4877)
(D) OTHER INFORMATION:/label= PTA29
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(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:4924..5216
(D) OTHER INFORMATION:/label= Pnos
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(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:5217..5489
(D) OTHER INFORMATION:/label= barstar
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(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:5490..5765
(D) OTHER INFORMATION:/label= 3'g7
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(A) NAME/KEY: -
(B) LOCATION:complement (5840..5864)
(D) OTHER INFORMATION:/label= LB
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CLAIMS

1. A plant having in the nuclear genome of its cells foreign DNA comprising

- a male-sterility gene comprising:
  - a male-sterility DNA encoding a sterility RNA, protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell, and,
  - a sterility promoter directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of said plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter; and
- a coregulating gene comprising:
  - a coregulating DNA encoding a coregulating RNA, protein or polypeptide which is capable, when produced in plant cells wherein said sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of said sterility RNA, protein or polypeptide, and preferably
  - a promoter directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells, or
  - a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is under control of enhancer elements in the nuclear genome of said plant.

whereby said coregulating DNA is in a transcriptional unit which is different from the transcriptional unit of said sterility DNA.

2. A plant according to claim 1, wherein said male-sterility gene and said coregulating gene are adjacent to one another.

3. The plant of claim 1 in which said sterility DNA encodes barnase or a variant thereof and said coregulating DNA encodes barstar. or a variant thereof.

4. The plant of any one of claims 1 to 3 in which said sterility promoter is PTA29 and said coregulating promoter is Pnos.

5. The plant of any one of claims 1 to 4 in which said plant is a dicot plant, particularly a Brassica plant.

6. The plant of any one of claims 1 to 3 in which said sterility promoter is PCA55, PE1, PT72 or PT42, and said coregulating promoter is P35S.

7. The plant of any one of claims 1 to 3 or 6 in which said plant is a monocot plant, particularly corn or rice.

8. The plant of any one of claims 1 to 3 in which said coregulating promoter is a minimal promoter operable in plant cells.

9. A cell of a plant according to any one of claims 1 to 8.

10. A process to obtain male-sterile plant which comprises :

- transforming the nuclear genome of plant cells with a foreign DNA comprising a male-sterility gene comprising:
  - a male-sterility DNA encoding a sterility RNA, protein or polypeptide which, when produced or overproduced in a stamen cell of the plant, significantly disturbs the metabolism, functioning and/or development of the stamen cell, and,
  - a sterility promoter capable of directing expression of the male-sterility DNA selectively in specific stamen cells, especially in anther cells, particularly in tapetum cells, of said plant, the male-sterility DNA being in the same transcriptional unit as, and under the control of, the sterility promoter, and
- regenerating plants transformed with said foreign DNA from said transformed cells, which method is characterized by including in said foreign DNA a coregulating RNA,

protein or polypeptide which is capable, when produced in plant cells wherein said sterility RNA, protein or polypeptide is produced, of sufficiently preventing the activity of said sterility RNA, protein or polypeptide, said coregulating DNA preferably being under the control of a promoter including:

- a promoter capable of directing expression of said coregulating DNA in non-stamen cells, preferably at least in the majority of non-stamen cells, while directing low-level expression, preferably not directing expression, in said specific stamen cells, or
- a promoter consisting of a minimal promoter element, preferably of a promoter normally expressed in plant cells, particularly whereby said coregulating DNA is capable of being placed under control of enhancer elements in the nuclear genome of said plant after integration of said foreign DNA in said plant genome,

whereby said coregulating DNA is in a transcriptional unit which is different from the transcriptional unit of said sterility DNA, and provided that, when said coregulating DNA is not under control of a promoter capable of directing expression in plant cells, said coregulating gene is located in said foreign DNA in such a way that after insertion in the plant genome, the coregulating DNA is capable of being placed under the control of plant promoter sequences present in the DNA surrounding said foreign DNA in said plant genome.

11. The process of claim 10 in which said sterility DNA encodes barnase or a variant thereof and said coregulating DNA encodes barstar or a variant thereof.

12. The process of claim 10 or 11 in which said sterility promoter is PTA29 and said coregulating promoter is Pnos.

13. The process of any one of claims 10 to 12 in which said male-sterile plant is a dicot plant, particularly a Brassica plant.

14. The process of claims 10 or 11 in which said sterility promoter is PCA55, PE1, PT72 or PT42, and said coregulating promoter is P35S.

15. The process of claims 10, 11 or 14 in which said male-sterile plant is a monocot plant, particularly corn or rice.

16. The process of claim 10 or 11 in which said coregulating promoter is a minimal promoter operable in plant cells.

17. A plant obtained by the process according to any one of claims 10 to 16.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PC1/EP 96/00722

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N5/10 A01H1/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,91 09957 (DU PONT) 11 July 1991 see example 12 ---	1-17
A	WO,A,93 19188 (MAX PLANCK GESELLSCHAFT) 30 September 1993 cited in the application see page 13, line 8 - page 14, line 27; examples 3,4 ---	1-17
A	WO,A,92 21757 (PLANT GENETIC SYSTEMS NV) 10 December 1992 cited in the application see claim 6 ---	1-17
A	WO,A,93 10251 (MOGEN INT) 27 May 1993 see the whole document --- -/--	1-17

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 May 1996	07. 06. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Maddox, A

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,93 08291 (PLANT GENETIC SYSTEMS) 29 April 1993 see page 10, line 9 - line 25 see page 14, line 7 - line 24 -----	1-17

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 96/00722

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